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THE EFFECT OF SODIUM HYDROXIDE ON SPORES
OF BACILLUS SUBTILIS, AS DEMONSTRATED BY
THE ELECTRON MICROSCOPE

by

JEROME F. PAULS

A THESIS

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The undersigned certify that they have read, and
recommend to the Faculty of Graduate Studies for acceptance,
a thesis entitled

THE EFFECT OF SODIUM HYDROXIDE ON SPORES
OF BACILLUS SUBTILIS, AS DEMONSTRATED BY
THE ELECTRON MICROSCOPE

submitted by Jerome F. Pauls in partial fulfilment of the
requirements for the degree of Master of Science.

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ABSTRACT

The effects of strong sodium hydroxide solutions on spores of Bacillus subtilis have previously been assessed, by the plate colony count method, by Whitehouse (1961) and other workers. It was hoped that direct examination, using the high resolution of the electron microscope, would make available new facts which could be related to the findings of these other workers. Accordingly, spores of B. subtilis were exposed to sodium hydroxide solutions under test conditions similar to those used by Whitehouse. Plate colony counts were made to supplement information given by the examination of whole mounts and sections of spores by electron microscopy.

Spores were exposed to solutions of 1.5% and 5.0% sodium hydroxide for from 12 to 72 hours; at temperatures of 50° and 70°F. Survivors were enumerated by plate colony counts on starch-skim milk-agar (SMA). Samples were mounted in Epon or Vestopal-W epoxy resins; sections cut and mounted on carbon coated grids. Whole mounts were made of phosphotungstic acid "stained" spores, on carbon coated grids.

The results indicate that sodium hydroxide solutions with an initial concentration of 1.5% rendered the spores non-viable by denaturation of the protein in all structural layers, disrupting the

cortex most rapidly. For exposure times of up to 13 hr., a 5% solution of the disinfectant appeared to prevent germination by changing the permeability of the spore wall, without any obvious structural damage; longer exposure resulted in gross destruction of the spore.

It was concluded that further investigation of the effects of sodium hydroxide, particularly early in treatment while the percentage kill was low, would be necessary before this method of studying damage to spores could be fully assessed.

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THE EFFECT OF SODIUM HYDROXIDE ON SPORES

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THE ELECTRON MICROSCOPE

INTRODUCTION

A study of disinfection processes may yield information leading to more effective and efficient methods of the use of disinfectants or may provide new knowledge about the life and death processes of bacterial cells. In the present study it was desired not only to show the gross effects of sodium hydroxide solutions on bacterial spores but, by varying such factors as temperature, concentration of the disinfectant solution, and contact time, to show possible differences in degree of damage to the bacterial spores, and perhaps the nature of the damage.

Disinfection

Chemical disinfectants act by creating adverse conditions either indirectly by poisoning, e.g. inactivation of essential enzyme systems, or directly, by destroying portions of the cell wall or cytoplasmic membrane.

Before chemical disinfectants can act they must make contact with the cell wall and, perhaps, penetrate through the cell membranes into the cytoplasm.

The major factors affecting the rate of a disinfection process are time, temperature, concentration of disinfectant, concentration of bacteria, pH, amount of non-living organic matter present, nature and age of the organisms involved, whether such organisms are on a surface or in suspension, and the effect, which may be additive or antagonistic, of other cations and anions which may be present. The medium used for growing the organisms may also affect their susceptibility to a disinfectant. These factors are discussed more fully below.

Contact Time

Disinfection is a gradual process which may take from a few seconds to a few days. Time is required for the disinfectant to penetrate the environment of the cell and then to penetrate the cell membranes. Individual cells will vary in their resistance to a given disinfectant, thus the greater the contact time the greater the extent of destruction of the bacterial population.

Temperature

Chemical disinfectants, as any other chemicals, are influenced by temperature, their rate of reaction increasing as the temperature rises. In disinfection of a bacterial population the temperature effect may be very complex, however, depending on the nature of the micro-organism as well as that of the disinfectant, and varying with each test organism. High temperatures in disinfectant solutions may cause destruction of bacteria by heat-coagulation of their protein,

rather than by chemical reaction. The degree to which this might occur will vary with the heat resistance of the organism.

Tilley (1942), using temperatures 10 C degrees apart, derived a temperature coefficient of 5 for phenol activity, on S. aureus, which held over the range of 10° - 50° C. This is of the same approximate order as the Q_{10} effect of common chemical reactions which is ca. 2.5 - 3.5.

Formalin and chlorinated lime act by releasing, respectively, gaseous formaldehyde and gaseous chlorine. An increase in temperature will increase the rate at which the gases are evolved, but unless the system is closed, it will also markedly increase the rate of loss to the atmosphere.

Most types of bacterial spores are extremely heat resistant. This resistance may reduce the apparent effect of temperature changes on a sporicidal disinfectant by eliminating heat-denaturation as a factor.

Concentration of Disinfectant

Usually, an increase in the concentration of a disinfectant will increase the rate of disinfection. However, the effect is not necessarily in direct proportion. The concentration exponent of phenol, for example, is given by Reddish (1957) as 6, and Levine (1952) noted that a 50% reduction in concentration of the sodium hydroxide solutions resulted in an increase in killing time for spores of Bacillus species of 240 - 300%.

With most disinfectants there is a "threshold" value above which an increase in concentration of the disinfectant will not result in an increase in its destructive power. In fact, 50 - 70% ethanol disinfects at a faster rate than stronger solutions under some circumstances. It has been suggested that this phenomenon results from the stronger solutions of ethanol (which, like other alcohols, acts by coagulating protein) forming a layer of coagulated protein around the outside of clumps of bacteria, protecting the innermost organisms, while the less concentrated solutions permeate, and eventually kill all of the organisms, having failed to cause sufficient peripheral coagulation to render the clump impermeable to further penetration.

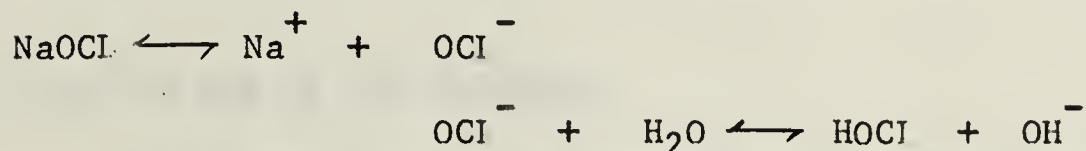
Concentration of Organisms

Total extinction time is extended as higher concentrations of organisms are exposed to a disinfectant. The usual reason for this effect is because the greater the number of cells the greater is the chance that the population will contain more resistant individuals, thus prolonging total extinction time.

Effect of pH

The effect of pH depends largely on the nature of the disinfectant. It is readily apparent that an environmental pH which reduces acidity or alkalinity of added acids or alkalis which depend on H^+ or OH^- ion concentration for their germicidal effects, will reduce the effectiveness of such compounds.

Less apparent, however, is the dramatic effect of pH on some other disinfectants, notably the hypochlorites. Reddish (1957) stated that the active germicidal agent of hypochlorites is free hypochlorous acid, formed as follows: e.g. sodium hypochlorite.



This hydrolysis is depressed with increasing alkalinity. Reddish presented a table which shows that, at pH 4.0 hypochlorite is present as undissociated HOCl to almost 100%; at pH 7.0 the hypochlorite is still undissociated to the extent of almost 70%, while at pH 10 the undissociated HOCl represents only 0.2% of the hypochlorite in solution. Alkaline solutions of sodium hypochlorite still possess some germicidal properties. This is probably due to the chlorine atom of the OCI^- ion.

Presence of Organic Matter

The presence of extraneous organic matter usually reduces the effectiveness of a disinfectant since most disinfectants work by combining with protein, without discriminating between living and non-living material. A familiar example is the "chlorine demand" of water, in which the amount of chlorine lost by combining with inert organic matter must be determined before the amount necessary for effective disinfection of the water can be calculated.

Large particles of organic matter may, thus, absorb enough of the disinfectant molecules to dilute its strength appreciably. Small particles of organic matter may coat the cells, or encourage clumping

of the cells - in either event hindering penetration of the disinfectant, although this effect will depend largely on the nature of the disinfectant.

Type and Age of the Organism

Different types of organisms vary in their resistance to disinfectants. Young, actively growing organisms are usually more susceptible than mature, or resting forms of the same species. Generally Gram-positive bacteria, and those containing a high lipid content, or those which form heavy slime layers or capsules are more resistant to disinfectants than are Gram-negative or non-capsulated types. Spores are the most resistant forms of bacterial life to disinfectants, as well as most other lethal agents, because of their resistant construction and relatively inactive metabolism.

Nature of the Supporting Surface

Depending on the nature of the disinfectant, and particularly on its possession or lack of detergent or surface-active properties, the physical environment of the micro-organisms to be attacked may be important. Surface-active disinfectants, for example, will not distinguish between bacteria and the surfaces supporting them. Surfaces are also generally more difficult to disinfect because the organisms involved are usually afforded some protection by films of organic matter. Chemical disinfectants usually enter the cell via the aqueous

phase (Sykes, 1958) and are, therefore, most active in aqueous solutions. Fat soluble disinfectants could have their effectiveness reduced by the presence of lipid material.

Other Cations and Anions

The effect of cations and anions other than those contributed by the disinfectant, will depend mostly on the nature of the disinfectant, although various ions do exert independent effects on the permeability of cell membranes, and thereby, on ease of penetration of extracellular solutes and solvents.

Sykes (1958) noted an increased germicidal effect for phenol in saline solution rather than water and notes a parallel with work of others in which it was found that addition of sodium or calcium chloride to soap and phenol-soap caused enhanced activity. This occurred with both cationic and anionic soaps and was attributed to their greater ease of diffusion and penetration into the bacterial cells, induced by lowering of their surface tensions.

Surface active compounds may be cationic, anionic, or nonionic, but the cationic group containing the quaternary ammonium compounds (QACs) is the most important group. The QACs are good wetting agents and act as germicides by protein denaturation and by affecting cell membrane permeability, however, they exhibit incompatibilities with certain other compounds. In particular they are completely inactivated by anionic compounds, including soaps; and by phospholipids, toward which they orientate their hydrophobic end.

The rate of disinfection by hypochlorites may be reduced substantially by the presence of ammonia, with the formation of chloramines. While these compounds have germicidal properties of their own, their action is much slower than that of the hypochlorites.

The presence of salts may reduce dissociation of an acid or an alkali, reducing the amount of H^+ or OH^- ion available for disinfection, without contributing any toxic element of their own, or the cation may itself contribute to disinfection, e.g. Ba^+ of barium salts.

Effect of Growth Medium

A growth medium may affect the outcome of a disinfection process both by its contributions to initial resistance of the microorganism and by its effect on recovery of partially damaged cells by supplying metabolites essential to that recovery.

Sykes (1958) noted that fatty acids in the growth medium acted to increase resistance, to heat, of subsequently formed spores by increasing their lipid content. Many workers have noted the relationship between sufficient calcium levels during sporulation and subsequent heat-resistance in bacterial spores.

Partially damaged bacterial cells may have lost the ability to synthesize some essential metabolite. Unless the medium supplies suitable intermediates or end products such cells will die. To estimate the number of spores surviving a killing influence it has become common practice to add germination stimulating substances such

as soluble starch (Williams, et al., 1957 a), or various amino acids to the recovery medium.

Methods of Studying the Action of Disinfectants

The action of a disinfectant can be examined by determining the number of survivors or observing the condition of damaged cells. Destruction can be evaluated either by determining total extinction times, or time-survivor curves.

The total extinction time is the time required to kill the entire population of organisms. The presence of even a few highly resistant organisms can, therefore, result in an extinction time which is not an accurate indication of the general efficiency of the disinfectant involved.

The time-survivor curve provides information on the rate of kill and provides definite end points, e.g. 99% kill, 99.9% kill, 99.99% kill, etc. Such information is useful, and not subject, to the same extent as total extinction results, to errors resulting from the existence of a few resistant cells. The shape of the curve may also provide useful information.

In a cytological study attempts may be made to deduce the method of attack and kill of a disinfectant from the observable changes in the cells gross and fine structure, staining reaction, refractive index, etc. Chaplin (1952) treated bacteria with QACs and attempted to relate the killing action of the disinfectant to changes in the morphology of the cells. After exposing vegetative cells and spores of

B. stearothermophilus to "Roccal" (alkyldimethylbenzylammonium chloride) he noted changes in cell walls and cytoplasmic membranes of the vegetative cells, and also apparently unchanged spores. Such studies might be made even more meaningful by using the increased resolution and magnification available in the electron microscope.

Types of Disinfectants

It is not possible or desirable in this account to list or discuss all the types of disinfectants in present use. However, it is desirable to consider the properties of a few main groups in order to justify the selection of the disinfectant used in this work. The four groups chosen for discussion differ markedly in their disinfectant and detergent properties.

Phenolic Compounds. Phenol (carbolic acid) is the classic example of a germicide and some of its substituted derivatives are actively used in industry today.

The free hydroxyl group of phenol appears to be the reactive moiety (Sykes, 1958). On absorption phenol forms a complex with the cell protein; this complex may ultimately coagulate. Depending on the concentration of the phenol, various modes of action may be expressed. In high concentrations the phenol will exert its killing effect by penetrating the cell and precipitating cytoplasmic proteins. At lower concentrations death of the bacteria results from phenol rendering the cell wall permeable to essential cell constituents, and at the lowest

lethal concentrations phenol appears to act by deactivating specific enzymes.

Soap-based disinfectants containing a high percent of phenol are available. Phenol itself has virtually no detergent properties.

Chlorine and Chlorine Compounds Free chlorine, handled as a compressed gas (liquid form), solutions of calcium and sodium hypochlorite, and the various organic chlorine compounds are probably the most commonly used disinfectants. The disinfectant activity of this group depends on the release of chlorine into the bacterial environment, its penetration of the cell, and the subsequent combination of the Cl^- or HOCl ions with constituents of the cell protoplasm. McCulloch (1945) has stated that the cell membrane appears to be particularly sensitive to chlorine, and that bacteria exposed to it tend to disintegrate. There has been insufficient study of the results of chlorination, however, to make a very positive statement about the primary site of chlorination damage.

Like phenol, chlorine has no detergent properties of its own, but it can markedly improve the germicidal effect of detergents on addition to them.

Quaternary Ammonium Compounds (QACs) The QACs are a group of disinfectants characterized as a group of amines which give nitrogen compounds with a valence of 5. Up to 4 of the covalent bonds are shared with alkyl or phenol groups, at least one being an alkyl group containing

C₈ - C₁₈ chains (Sykes, 1958). These latter groups contribute the property of surface activity, or detergency to these compounds. The QACs, although referred to as "cationic detergents" are poor detergents at the concentrations they are used as germicides (Sykes, 1958) but relatively good wetting agents.

There are various theories concerning the disinfecting action of QACs, with, no doubt, additional differences concerning individual compounds, but generally it is considered that death results from cell rupture caused by the surface activity of the QACs, interference with oxidation of cell metabolites at an intracellular level, and interference with cell membrane permeability with a subsequent loss of cell nitrogen and phosphorous.

Being surface active the QACs are readily adsorbed on any surface. In practical disinfection this means that treated surfaces are left with a residual layer of the compound which can continue to exert its germicidal effect. It also means, however, that any non-living particulate matter present can remove substantial amounts of a quaternary from solution.

Sodium Hydroxide The disinfectant properties of sodium hydroxide are commonly believed to be due to the OH⁻ ions, which are known to denature protein by hydrolysis (Rahn, 1945; McCulloch, 1945; Foster et al. 1957). Levine (1952), however, noted the following:

- (1) addition of sodium chloride to solutions of sodium

hydroxide increased their toxicity, although clearly reducing dissociation and OH^- ion concentration

- (2) different alkalies, e.g. KOH, NaOH, at the same pH, showed different sporicidal effects, although being dissociated to the same degree (same OH^- ion concentration).

From these observations Levine suggested that, in some bacterial spores at least, the concentration of undissociated sodium hydroxide entering the organism might be the controlling factor in disinfection.

Sodium hydroxide is normally used as a detergent but, although relatively slow in action, it is an effective disinfectant because of its high degree of dissociation and subsequent high OH^- ion concentration. It is less sensitive to the presence of non-living organic matter than are most disinfectants (McCulloch, 1945), a factor favoring its use in cleaning food processing equipment with their residues of organic matter.

Rate of Disinfection

If a disinfection process is to be studied, rather than the determination of an end result, it will be desirable to slow down the rate of reaction. This suggests the use of a relatively slow acting disinfectant, such as sodium hydroxide, at a low concentration, and with a resistant micro-organism such as a spore former. By varying the concentration of the disinfectant and the reaction temperature the rate of disinfection can be controlled within fairly narrow limits.

Such disinfection systems have been developed and studied previously by several workers, however, they were interested in determining percent survivors under practical disinfection conditions rather than in observing the effect of the disinfection process on the individual cells.

SPORES

Bacterial spores, unlike their vegetative counterparts, exhibit a very low metabolism and a dense, resistant structure. The protection afforded their sensitive enzyme systems renders the spores far less vulnerable than vegetative cells to such agents as heat, desiccation, exposure to ultra-violet irradiation and chemical disinfectants. Spores of Bacillus subtilis have shown the efficiency of this survival-orientated construction and metabolism by surviving, in soil samples, for over 300 years (Sneath, 1962).

In order to observe and interpret possible damage to spores it is necessary to be familiar with current concepts of spore properties and structure, including the processes of sporulation and germination, and changes which can occur in spores as a result of natural death or experimental handling.

Although much study has been given to the initiation of sporulation the phenomenon is still only partially understood. While healthy cells, facing starvation, will normally sporulate so also will cells whose nutrition appears to be adequate.

Cytology of Sporulation

The Participation of the Vegetative Cell Understanding the process of sporulation depends, in part, on knowledge of the structure and chemistry of the vegetative cell in which the spore develops. Current concepts of fine structure, chemical composition, and participation of some structures in sporulation, of vegetative cells of Bacillus subtilis, and, where more information is available, of other Bacillus species, form the following section.

The Cell Wall Vegetative cells of B. subtilis are bound by a cell wall which comprises a double layer of electron dense material divided by a less dense layer or area (Tokuyasu and Yamada, 1959 a; Glauert et al. 1961; Kawata et al. 1963). The outer and inner layers are each about 40 A° thick, and the intervening layer from 120 to 170 A° thick.

Glauert et al. (1961) report that Ryter and Kellenberger have suggested that the two dense layers are, themselves, double. Glauert's group disagree with this interpretation, suggesting that one layer of the cytoplasmic membrane has been mistaken for part of the cell wall.

The cell walls of B. subtilis, like those of other Gram-positive bacteria, contain few amino acids and relatively small amounts of lipid material. An amino acid analysis of the walls of vegetative cells and spores of B. subtilis has been presented by Halvorson (1962) and its content shown in Table 1. (see page 16)

Salton and Marshall (1959) analysed walls of vegetative cells of B. subtilis for their general chemical content. Some of their findings

are compared with data from an analysis of the general composition of spore walls of B. subtilis (Halvorson, 1962) in Table 2.

Table 1. The amino acid composition of cell and spore walls of B. subtilis (from Halverson, 1962).

Molecular Proportions of Amino Acids in:		
	Vegetative cells	Spores
lysine	0	6
glycine	0	5
serine	tr.	4
alanine	4	4
glutamic acid	2	3
val. / meth.	tr.	2
leuc. / isoleuc.	tr.	2
tyrosine	0	1.5
aspartic acid	0	1
diaminopimelic acid	1	1
threonine	0	1

tr = trace

Table 2. Some constituents (as % total dry weight) of the walls of vegetative cells and spores of B. subtilis (from Halverson, 1962).

Constituents, as % Total Dry Weight, in:		
	Vegetative cells	Spores
Total N	4.6	12.9 - 13.1
Total P	4.2	1.4 - 1.6
N-acetyl glucosamine	7.9	1.0 - 4.0
N-acetyl muramic acid	2.3	0 - 0.6
Lipid	0.7	1.1 - 3.0

Salton and Marshall (1959) concluded that the spore wall of B. subtilis is largely composed of structural protein, in contrast to the peptide - amino sugar - sugar complex of the walls of vegetative cells. These results support the proposal that spore wall protein is the result of de novo synthesis during sporulation. The only contribution of the vegetative cell wall to sporulation would appear to be to provide a limiting force against which the inward buckling spore septum can push (see below).

The Cytoplasmic Membrane The cytoplasmic membrane of B. subtilis is described by Glauert et al. (1961) as having electron dense outer and inner layers, separated by a less dense layer. The outer layer is 30-40 A° thick, the inner layer about 15 A° thick, and the intervening, lighter layer is about 50 A° thick.

Weibull and Bergström (1958) studied the chemical composition of the cytoplasmic membrane of B. megaterium, strain M. They found the membrane to consist of 63-69% protein, 16-21% lipid, and most of the rest as glucose, probably as a glycogen-like polysaccharide. Fitz-James (1960) suggests that the membrane exists as a phospholipoprotein structure. In the same paper this author suggests, although only as a speculation, that the dimensions and certain staining characteristics of the cytoplasmic membrane layers may be indicative of the molecular arrangement of this (phospholipoprotein) structure. These observations, and the work of Stoeckenius (1959) suggest that the cytoplasmic membrane may exist as a single, bimolecular leaflet

of phospholipid, bearing external layers of protein.

Mesosomes Pockets of membranous material which appear to be continuous with the cytoplasmic membrane have been studied by many workers (e.g. Chapman and Hillier, 1953; Chapman and Kroll, 1957; Glauert and Hopwood, 1959; Fitz-James, 1960; Kawata et al. 1963). Commonly referred to as mesosomes, but also described as membranous organelles and as intracytoplasmic membranous systems, they are found associated with the advancing edge of the septa in cross-wall and spore septum formation, and with the nuclear material of the cell. They appear to be convoluted intrusions into the cytoplasm in which proliferation of the tissues has created a multi-layered "organelle". Robinow (1962) has remarked on the likeness of mesosomes to myelin figures and noted that, like such figures, the lamellae of the mesosomes may be composed of bimolecular leaflets of phospholipid.

Nuclear Material The nuclear material of B. subtilis is centrally located, in the form of two multi-lobed masses (Young and Fitz-James, 1959 b). It is fibrillar in nature, existing as a network of fine strands (Kellenberger et al. 1958; Glauert, et al. 1961).

Sporulation Morphologically speaking, sporulation begins when the nuclear material of the vegetative cell fuses into a single, long axial thread with one end extending into the pole of the cell destined

to hold the future spore (Young and Fitz-James, 1959 b; Murrell, 1961). In this end of the cell the nuclear material forms a large, distinctive mass usually including, or in association with, a mesosome (Young and Fitz-James, 1959 b; Kawata et al. 1963). This region now becomes more defined by the actions of the cytoplasmic membrane.

Somewhat back from the appropriate pole of the vegetative "mother cell" the cytoplasmic membrane is reflected into the cytoplasm as a centripetal, or annular invagination. On completion this will form a transverse "spore septum" isolating the area of future spore development. The nature of this septum is indicated in Fig. 1. (see page 19).

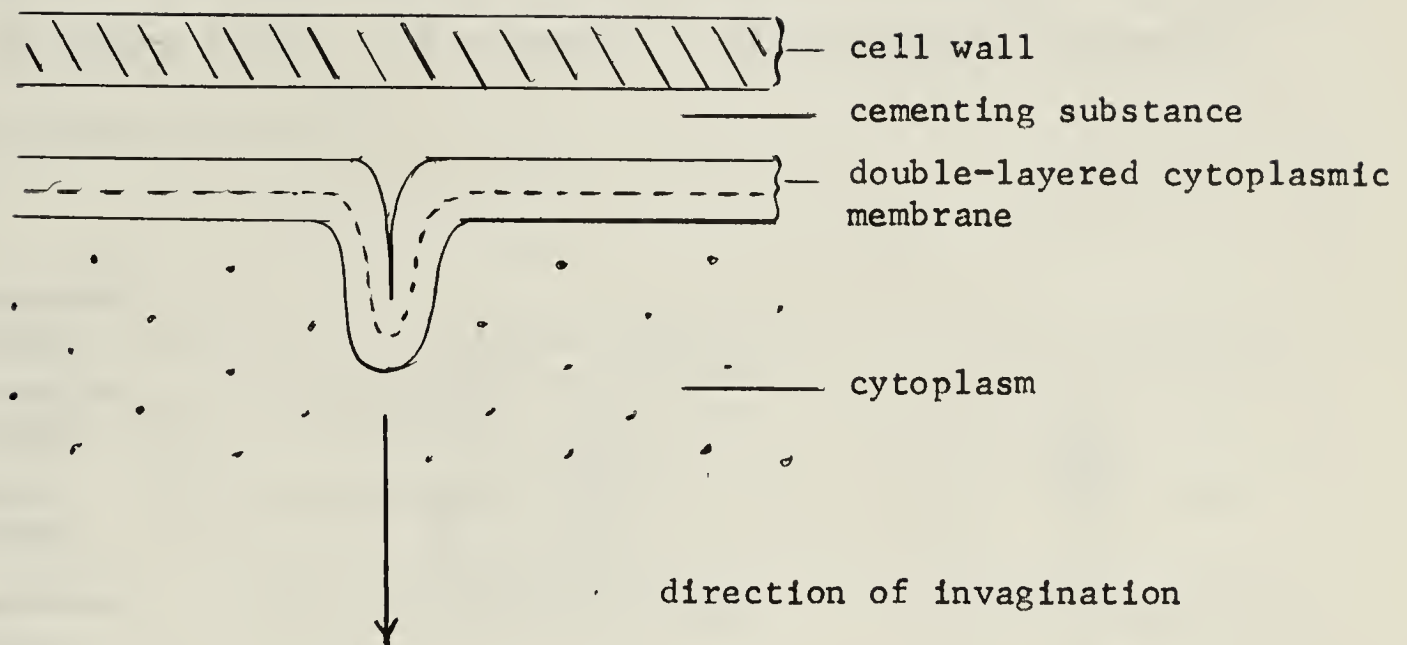


Fig. 1. Diagram of developing spore septum

(from Fig. 16, Fitz-James, 1960) - note that the septum will consist of two double layers.

The intruding edge of the septum is usually connected to one or more mesosomes (Chapman and Hillier, 1953; Tokuyasu and Yamada, 1959 b; Kawata et al. 1963). Fitz-James (1960) also mentioned this, and described how the septal membrane, via mesosomes, forms a connection with the portion of the nuclear mass destined to be incorporated into the spore, and eventually isolates it. On its completion the transverse septum begins to bulge, and develop more mesosomes, on the side distal to the spore field (Ohye and Murrell, 1962; Robinow, 1962). At this same time the edges of the septum proliferate around the cell end to form the initial spore envelope and complete a young forespore. The enclosed nuclear material takes up a peripheral position in the spore cytoplasm (Robinow, 1951). This cytoplasm, with its nuclear

material and enclosing septum will form the germinative "spore core" of the mature spore. The movements of the transverse septum are illustrated in Fig. 2.

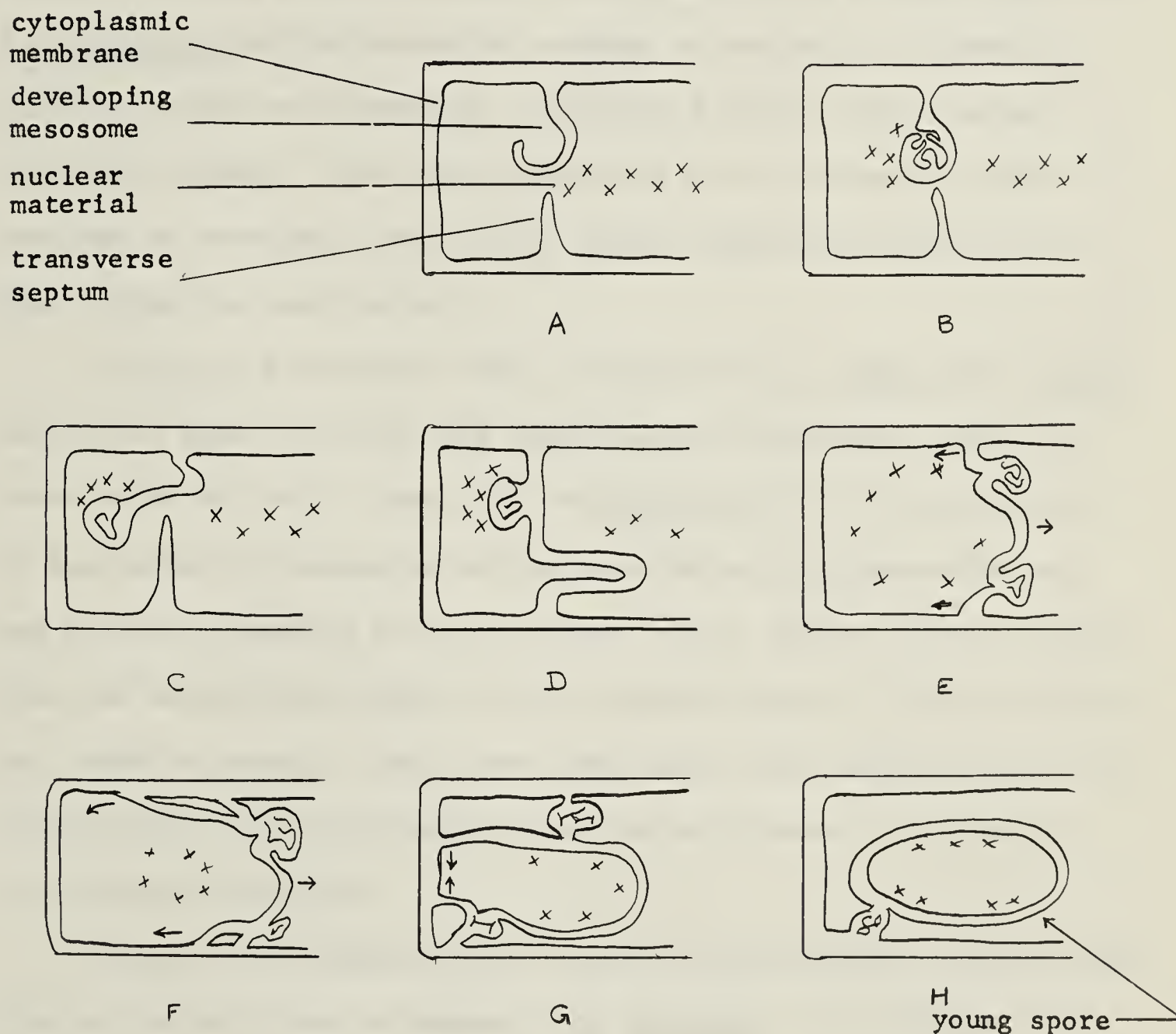


Fig. 2. Proliferation of the transverse spore septum in sporulation
(from Fig. 9, Fitz-James, 1960)

Between the two double layers of the septum surrounding the young spore (forespore) the cortical material now begins to be laid down

(Young and Fitz-James, 1962). Mayall and Robinow (1957) described the cortex as 'a special, intricately organized layer, intervening between the dormant bacillus and the spore coats.' These authors, viewing sections of acid treated, lanthanum nitrate stained spores of B. megaterium saw the cortex as a system of concentric strands, or lamellae, which are permeated, in untreated spores, with a second continuous phase. These same laminations were subsequently seen in sections of untreated spores of B. cereus, suggesting that they are real rather than artifactual.

Young and Fitz-James (1962), working with B. cereus var. alesti noted that when the cortex was about one third developed, the spore coats began to form. Those laid down peripherally to the outer edge of the cortex are assembled in the cytoplasm of the vegetative cell and are not, according to these authors and to Robinow (1962), derived from the outer double-layer of the forespore septum. Without disagreeing, Ohye and Murrell (1962) note that these outer layers of the forespore septum, while not synthesizing the outer spore coats, may be incorporated into them.

Bradley and Franklin (1958) describe the elaborate contours which form on the outer coat of spores of B. subtilis 'ribbed, ribs usually longitudinal and rather irregular, sometimes transverse at the ends of the spore, the extent of the ribbing varying with strains.'

The spore core coat(s) and cortical coats internal to the inner edge of the cortex arise directly from the inner double-layer of the

forespore septum (Young and Fitz-James, 1962). The mature spore is shown in Fig. 3.

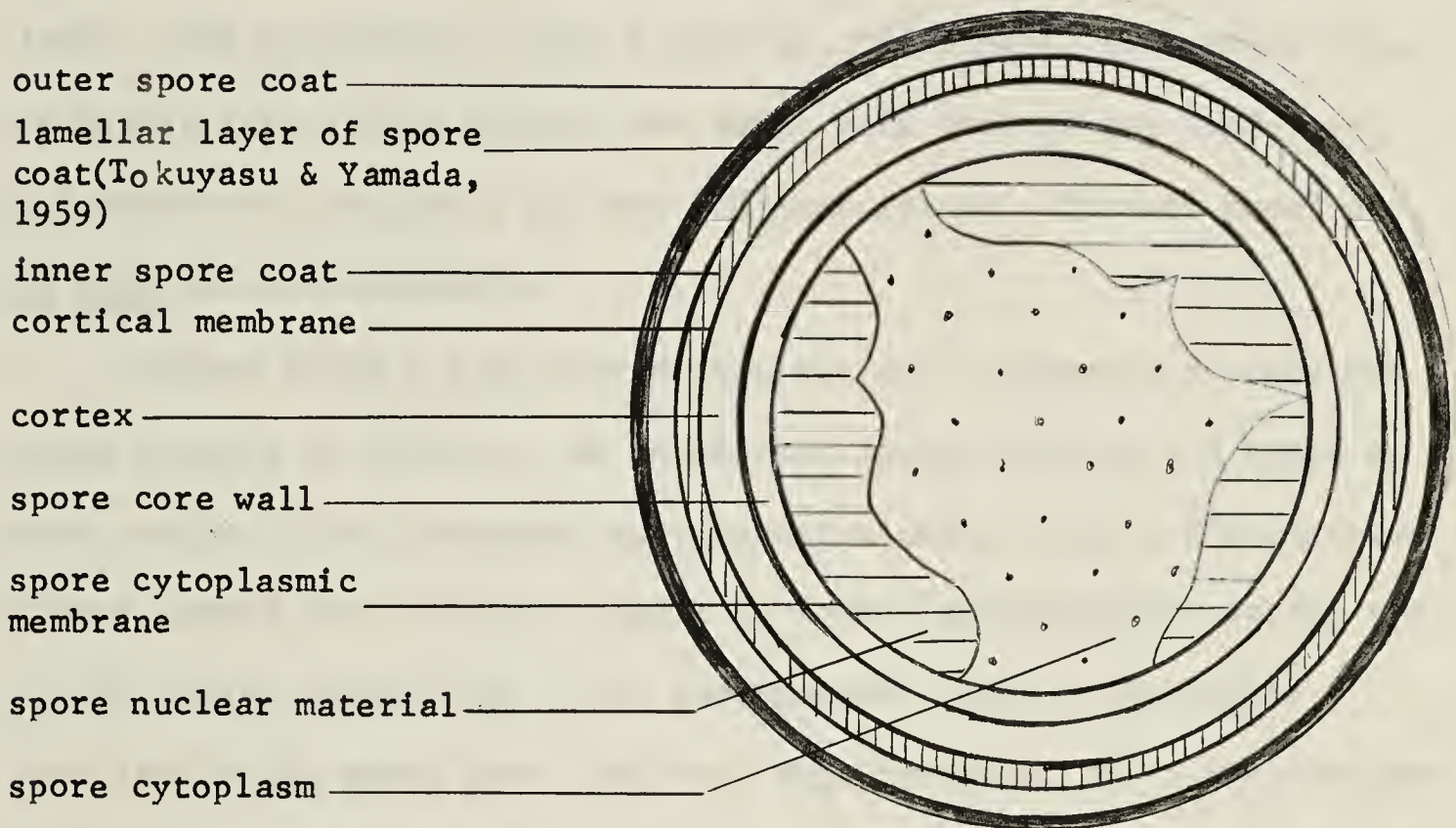


Fig. 3. Schematic diagram of a mature spore of B. subtilis

Chemical Changes in Sporulating Cells

The chemistry of sporulation, and of the spore itself, may give clues about the role the spore is designed to play. For example, the comparative analysis of spore and vegetative cell components made by Halvorson (1962) and by Salton and Marshall (1959), both previously referred to, indicate the extensive alterations which occur during sporulation, and offer some proof of the de novo synthesis of spore wall protein.

Foster et al. (1954) and Widra (1956) studied the movement of

protein-bound sulphhydryl groups during sporulation and considered the ultimate inclusion of these in the spore walls as a proof of de novo synthesis of spore protein. They, as well as others (Young and Fitz-James, 1959 b; Murrell, 1961; Halvorson, 1962) noted that sporulation parallels utilization of the free amino acid pool of the cells, and degradation of available purines and pyrimidines - further proof of de novo protein synthesis.

Vinter (1960 a & b) stated that the main component of this new spore protein is cystine. He noted that spores contain 2-5 times as much cystine (plus cysteine) as vegetative cells. Using ^{35}S -cysteine Vinter showed that the new protein is formed approximately during the "young spore" stage, that it is incorporated into the insoluble fraction of the spore coat, and that this occurs prior to the development of heat resistance by the spore.

Widra (1956), using differential staining techniques, concluded that the spore walls of some Bacillus species consist of a lipoprotein. He also suggested that within the lipoprotein there are lipid granules or inclusions.

Vinter (1960 b) used $^{45}\text{CaCl}_2$ to study incorporation of calcium into the spore. He found that incorporation occurred after formation of the new protein structures, and that thermal resistance developed at this time. Calcium uptake and the development of thermal resistance in spores is paralleled by a third development, synthesis of dipicolonic acid (DPA). Powell and Strange (1953) noted that this compound, which can form as much as 15% of the total dry weight of spores, is completely

lacking in the vegetative counterparts of such spores. It has been noted that DPA can be synthesized in the absence of calcium; the resulting spores being heat sensitive.

Riemann (1961) noted that calcium and DPA are present in the spore in a 1:1 molar ratio, and suggested that they exist as a calcium - DPA chelate.

Rode and Foster (1960) noted that the DPA could be rapidly removed from spores by mechanical rupture or boiling, and that the free form was a calcium salt. They suggested that DPA is present as a chelate, with calcium, without strong chemical bonding to any other spore substances. However, Harper et al. (1964), using electro - dialysis of whole spores, concluded that DPA in ungerminated spores is either strongly bound or is located in an anhydrous portion of the spore.

Attempts to locate the proposed chelate have been unsuccessful. Mayall and Robinow (1957) compared their sections of ungerminated and germinated Bacillus spores. They related loss of cortex with loss of DPA and suggested the cortex as the site of the chelate. Conversely, Hashimoto and Gerhardt (1960), using monochromatic ultra-violet microscopy, were able to see a sufficiently integrated cortex, after loss of DPA, to make them question the cortical site for the chelate.

Powell (1957) regarded the spore as being a highly condensed, waterproofed system stabilized by the incorporation of calcium dipicolonate, but did not suggest the location of the chelate. Worth et al. (1963) considered the cortex to be the storage site of carbohydrate. Hashimoto et al. (1960) were able to relate DPA synthesis

and development of the spore cortex, but heat resistance did not appear until late, when DPA concentrations were quite high.

These studies suggest that spore DPA (1) exists as a calcium salt, at least in the spore cortex (2) may not be limited to the cortex (3) may, above a minimum concentration, contribute to thermal stability by forming complexes with spore protein.

No respiration is normally observed in resting spores but enzyme activity has been demonstrated, with both whole spores and spore extracts. An alanine racemase [$L \xrightarrow{50} D$] in B. cereus var. terminalis (Stewart and Halvorson, 1953), a catalase (Lawrence and Halvorson, 1954), an adenosine deaminase [$\text{adenosine} \xrightleftharpoons{\text{HOH}} \text{inosine} + \text{NH}_3$] and a hydrolytic nucleotide ribosidase [$\text{adenosine} \xrightarrow{\text{HOH}} \text{adenine} + \text{ribose}$] (Powell and Hunter, 1956) have been isolated, which are heat stable and active in "dormant" spores, but heat labile when isolated. In addition, Bach and Sadoff (1962) found a glucose dehydrogenase in B. cereus which is heat resistant when isolated, and would seem to have a protected structure of its own.

The cortex is usually regarded as a sort of permeability barrier surrounding an anhydrous spore core. The nature and limits of this impermeable structure have not been well defined to date. Ross and Billing (1957), using phase contrast and interference microscopy, with protein immersion, thought that the refractive indices of their spores compared well with dehydrated protein, and suggested that spores contain very little water. Conversely Gerhardt and Black (1960), using B. cereus var. terminalis, found that spores took up glucose

to an extent of 51% of spore volume, or 40% of spore weight. This added glucose was easily washed out of the spores, leaving them still viable, thus the glucose appeared to enter the spores by simple diffusion. Lipid soluble materials such as ethylene glycol were observed to be taken up to a greater extent. It was noted that uptake increased in heat-killed (DPA free) spores. Using tritium-labelled water a free exchange of internal and external water, through the spores, was also demonstrated.

These investigations, while not conclusive of the possibility of an anhydrous spore core, do suggest that the cortex is not as impermeable as was previously thought. Further, the increased permeability of heat killed (DPA free) spores suggests that the calcium-dipicolonate chelate plays a part in controlling spore permeability as well as any function it may have in promoting thermal stability of protein.

Germination

In this paper germination will be discussed only as it relates to structure and function in the mature spore.

In a medium suitable for vegetative growth and/or after exposure to a germinating agent, spores of Bacillus species will take up water, swell, and undergo a variety of changes which culminate in the emergence of a growing vegetative cell. During germination the spore excretes its DPA as calcium dipicolonate. It also excretes a hexosamine peptide which Powell (1957) suggested came from the spores outer coats.

At this same time the spore cortex becomes less clearly defined. Robinow (1960) suggested that the cortex, at this time, is taking up water. The spore core coat, originally synthesized by the inner layers of the spore septum, now develops into the wall of the new vegetative cell (Robinow, 1960).

Theories of Spore Resistance

The thermal resistance of bacterial spores is probably an insignificant factor in their survival in nature. Because of its industrial importance and its relation to many studies on the nature of the calcium dipicolonate of spores, however, this property has been given more attention than the other resistant properties of the spore.

Many eminent workers (e.g. Powell, 1953; Grelet, 1957; Halvorson, 1957; Church and Halvorson, 1959; Vinter, 1960 a & b) are in agreement that the main factor in this thermal resistance is a calcium-dipicolonic acid chelate, which acts by stabilizing the otherwise heat-labile proteins of the spore. The location and mode of action of this complex are still unknown. Some evidence has been presented for its concentration in the spore cortex. Powell (1957) suggested that the chelate may be present throughout the spore protoplasm, where it contributes to the production and maintenance of heat-resistant properties. Powell further suggested that the integrity of the outer spore coats is important in maintaining the heat stable structure of the spore.

Halvorson and Church (1957) noted that the release of DPA, during germination, paralleled the activation of a glucose dehydrogenase. They suggested that the calcium-DPA, by binding such enzymes prior to germination, may contribute to their dormancy.

Vinter (1960 b) noted that the formation of the cystine-rich outer spore coats paralleled a marked increase in the spores resistance to ultra-violet radiation. He suggested that this is because of the ability of the protein to use the disulphides with attendant-SH groups, as a source of electrons to replace those knocked out by irradiation.

Doi (1960) suggested that the disulphide linkages described by Vinter may contribute to the dormancy of spore enzymes by masking their structure.

Widra (1956) and Salton and Marshall (1959) found lipid material in the walls of spores of Bacillus species. Curran (1952) stated that the cytoplasm of the spore is rich in diffusely distributed lipid material, which would account for much of the hydrophobic nature of resting spores. This also might contribute to the dormancy of spore enzymes by masking active sites, and would protect proteins by impeding the penetration of many substances and, to some extent by masking peptide linkages.

Electron Microscopy as a
Method of Studying Spores

Electron microscopy is a relatively young discipline. A result of this is that a newcomer to the field of electron microscopy finds only a few basic texts on the subjects of fixing, embedding, and otherwise preparing his specimens for inspection, and much of such information must be sought from reviews of current work. A disadvantage of this approach is that the reviewer will find that few experiments or techniques have been used sufficiently for unqualified reliance to be placed on them. This is especially true where additional experimental treatments, different from those of the original worker, are planned.

At the beginning of this work it was realized that there was no certainty of showing, in a useful way, the damaging effect of sodium hydroxide solutions on spores, by electron microscopy. It was felt, however, that in the event of a negative outcome it would be useful to show why this method of investigation was unsatisfactory.

Artifacts

Unlike studies using the light microscope, with its attendant rapid, well developed and relatively simple techniques, preparation of specimens for electron microscopy can be elaborate and time consuming. The opportunities for introduction of artifacts are numerous and their detection often difficult. This is especially true with the

present work in which the experimental treatments are expected to do some physical damage to the cells. For example, the rupturing of spores may be caused by exposure to sodium hydroxide or by polymerization damage, or as a result of faulty cutting technique or inadequate penetration of fixatives or embedding materials. Controls, replication, and experience make artifacts easier to recognize, but they may be subtle, and easily mistaken for the results of experimental treatments, in spite of safeguards.

Sources of Artifacts

Fixing Palade (1952) stated.. 'One of the most difficult problems in electron microscope cytology is to evaluate the quality of fixation, i.e. to decide how closely the fixed image approximates that of the living cell.' This worker reported that the pH of the fixative could be a critical factor in cell preservation, acid fixatives appearing to alter cell membrane permeability, with resultant loss or rearrangement of the cell contents.

Rode et al. (1962) noted the appearance of abnormal structures after fixing spores of B. megaterium with either KMnO_4 or OsO_4 . They concluded that the alterations were caused by solution and loss of spore contents, and that the fixatives did not penetrate well after encountering the spore cortex.

Holbert (1960) also noted that OsO_4 , unless precisely buffered, dissolved out some components of spores of B. polymyxa.

Several other workers (Robinow, 1953; Chapman, 1956; Fitz-James,

1959; Tokuyasu and Yamada, 1959 b) have noted similar artifact formation, and an explosion artifact, during fixation.

Dehydration Exposure to high concentrations of ethanol or acetone may disperse or rearrange delicate membrane systems and alcohol or acetone soluble components in bacterial cells. In bacterial spores, where fixation may be incomplete, lipid fractions may be removed.

Embedding Holbert (1960) notes that the accelerators used with epoxy resins appear to be slow in penetrating cells. If the difference in penetration rates of resin and accelerators is sufficient to result in some separation of the two, the resin, on polymerizing, will tend to shear or crack. This will distort, and even destroy, enclosed specimens.

Sectioning Hashimoto and Gerhardt (1960) and Robinow (1960) allude to loss of DPA during sectioning. Robinow states that the calcium salts of DPA may be dissolved in the water used to float newly cut sections. This could result in, among other things, an apparent change in the structure and volume of the cortex.

The angle of the cutting surface of the glass or diamond knife used for sectioning is very important to the quality of the resulting sections. Improper knife angles can result in sections bearing marks, or sections which have been distorted by stretching, shearing or

compression. All of these conditions may produce artifacts. Too severe a knife angle can also "enucleate" spores, that is, the spore core is lifted neatly out of the section, leaving the surrounding membranes relatively undisturbed.

Spore Preparations for the Electron Microscope

Because bacterial spores are so dense to electrons as to render them wholly opaque when viewed in an electron microscope, thin sections are normally used. Whole mounts may be useful where the spore has ruptured and lost sufficient of its contents to be somewhat less opaque to the electron beam, but the usefulness of such preparations is limited. One method of studying the topography of a spore, if sections are not suitable is by replicating and shadowing (i.e. Bradley and Williams, 1957).

MATERIALS AND METHODS

In order to apply the results of this study to practical disinfection studies (e.g. Whitehouse and Clegg, 1963), and because no other materials, methods, or organisms seemed especially recommended over them, it was decided to investigate the effects of sodium hydroxide solutions on spores of B. subtilis. The concentrations, exposure times, and temperatures of practical applications in immersion cleaning of milking equipment were used i.e. sodium hydroxide concentrations of 1.5-5.0% (w/v), temperatures of 32-80° F., and time periods of up to 72 hr. were considered.

The method of exposing the spores to the disinfectant was that of Whitehouse and Clegg (1963), which is a modification of the method of Franklin and Clegg (1956).

The spores used were strains of Bacillus subtilis (NIRD 736 and SM 761). These were the types of organisms used in the immersion cleaning trials referred to above.

Choice of Disinfectant and Test Organism

Sodium hydroxide was chosen as the disinfectant because

- (a) it can be used to produce a slow disinfection process
- (b) in order to more readily relate the results of this work to recent studies by Whitehouse (1961), and to earlier workers.

Bacillus subtilis spores were chosen because

- (a) use of the resistant spores aids in slowing down the disinfection process
- (b) in order to aid in relating the results of this work to immersion cleaning studies in which solutions of sodium hydroxide were used against such spores
- (c) since bacterial spores present interesting and industrial-ly significant problems because of their high resistance to routine disinfection techniques and this study might contribute to a better understanding of some aspects of this resistance
- (d) because B. subtilis spores appear to be intermediate, as representatives of Bacillus species, in their resistance to sodium hydroxide (Gehuchte, 1962).

Treatment of Test Organism

Preparation of Spores

Freeze-dried spores of B. subtilis strain SM 761 were obtained from the National Collection of Dairy Organisms, National Institute of Research in Dairying, University of Reading, and specimens of B. subtilis strain NIRD 736 which had been frozen-dried during the course of past work (Whitehouse, 1961) were obtained from within the department. Both were grown out in B.B.L. trypticase soy broth at 37° C for 24 hr., and cultivated on a solid medium used by Whitehouse (1961) adapted from

Williams et al. (1957 a). This medium contained manganese to stimulate spore production and was made up as follows:

Bacto tryptone	3g
Bacto peptone	6g
Yeast Extract	3g
Beef Extract	1.5g
Agar	25g
Solution containing	
0.001% Mn as MnSO ₄	1 ml.
Distilled water to	1 litre

pH approx. 7.0

The cultures, in Roux bottles, were grown for 7-9 days at 37°C, until sporulation was virtually complete. Microscopic examinations at this time normally revealed only a dozen or so vegetative cells in a field containing several hundred spores. The spores were removed from the agar surface using a wash of sterile distilled water, and sterile glass rod scrapers. The suspensions so formed were decanted into sterile screw-cap bottles containing Fisher 3mm. glass beads, and these shaken for 3 hr. to reduce clumps. The suspensions were then heated at 80° C for 20 min. to kill any vegetative organisms present.

The suspensions were cooled to 4°C to minimize spore germination, and washed with pre-cooled distilled water in a refrigerated centrifuge. Stewart and Halvorson (1952) found that repeated washing was necessary for cleaning spores; since very clean suspensions are required for photography a modification of the method of Long and Williams (1958), described below, was used.

Spores, vegetative cells, and debris were first centrifuged at 12,000 x g for 10 min. The supernatant fluid was examined, found to be

free of spores, and removed by pipetting. The pellet of cells was resuspended in distilled water, and again centrifuged as above. Examination of the packed material now revealed a light gray layer of vegetative cells overlaying a creamy white spore layer which, in turn, was overlaid on a small amount of brown debris. The centrifuge tubes were held at an angle, approximately 5 ml. of distilled water was pipetted into each tube and the tube gently rotated. This caused the vegetative layer to come away from the underlaying spore layer in large, viscous pieces without the spore layer being disturbed. Microscopic examination of the dislodged material showed mostly vegetative cells and few spores, and this material was discarded. After three such washings a well defined vegetative layer could no longer be seen. Three further washings removed most of the dark, heavy debris, and microscopic examination revealed a clean and almost vegetative cell-free spore preparation.

The cleaned spore suspensions were stored at 4°C until required. Daily checks with the light microscope showed a clean and apparently stable spore crop with no vegetative cells appearing and spores remaining uniform in size and staining reaction to crystal violet. When examined in a phase-contrast microscope all spores appeared highly refractile.

Disinfection and Recovery Procedures

Exposure to Disinfectant

The method of Whitehouse and Clegg (1963), based on earlier work

by Franklin and Clegg (1956), was used. Ninety ml. quantities of control and disinfectant solutions, in sterile 300 ml. Erlenmeyer flasks, were allowed to equilibrate in a refrigerated water bath for 24 hr. before inoculation. Sterile glass beads were included to facilitate thorough mixing. Cotton wool closures covered with aluminum foil were used during the longer trials to reduce loss of solution by evaporation.

These solutions were inoculated with 10 ml. quantities of the spore suspension, which had also been equilibrated to the temperature of the test solutions in order to avoid shocking the spores with an extreme temperature change on inoculation. The initial concentrations of the test solutions were such that the addition of 10 ml. of spore suspension gave the desired final concentration of sodium hydroxide.

Recovery of Treated Spores

After inoculation and thorough mixing and at selected time intervals, 10 ml. samples were taken of the spore-disinfectant suspension. These were immediately added to tubes containing 10 ml. of hydrochloric acid (of the same normality as the sodium hydroxide), 10 ml. of sterile phosphate buffer (at approximately pH 7.0), and 2-3 drops of sterile brom-thymol blue indicator. These acid - buffer - indicator mixtures had also been equilibrated for 24 hr. at the temperature of the test and control solutions. The brom-thymol blue gave a fast visual check on the neutralization of the alkali.

After neutralization, 2 ml. of the sample was taken for duplicate

plating on SMA (see below). The remaining solution was used for the electron microscope studies.

Enumeration of Spores Surviving Disinfection

The nature of the electron microscope limits the size of a sample of a bacterial population which can be studied to a relatively small number of individual organisms. To say that such a sample is representative of the whole population, therefore, requires some secondary sources of knowledge about the condition of that population; enumeration of survivors by the plate colony count is one such source.

Spores such as those treated here may, after exposure to the sublethal agent, require special stimulation before they will germinate (Curran and Evans, 1937; Franklin and Clegg, 1956; Amaha and Ordal, 1957; Williams et al. 1957 a & b). Whitehouse (1961) evaluated several recovery media, and after reviewing his findings it was decided to use a recovery medium of starch - milk - agar (SMA) i.e. nutrient agar containing skim milk (1% v/v) and soluble starch (0.1% w/v).

Preparation of Spores for

Electron Microscopy

Preparation of Whole Mounts

Thin sections of spores show internal structure but give little indication of surface detail. If, for example, exposure to sodium hydroxide caused rupture of the spores, and if this rupture usually

occurred along a certain axis of the spore or at the poles, thin sections might not indicate this clearly. Consequently, examination of whole mounts seemed indicated.

Knaysi et al. (1947) noted that because of the high density of spores to the electron beam, direct examination of whole mounts in the electron microscope yields only outlines of the spores. From this, the most obvious approach seemed to be that of replication e.g. the carbon replication method used by Bradley and Williams (1957).

Because of technical difficulties with the available equipment, replicating, while not impossible, was difficult enough to warrant further investigation of whole mounts. The opacity of spores to the electron beam is reduced if they are ruptured and lose part of their contents. Therefore, it seemed that information might be obtained from studying whole mounts where rupture points were of interest and where ruptured spores were expected. It was also thought that negative staining with phosphotungstic acid (PTA) according to the method of Abram (1965) might help to demonstrate external membranes in whole mounts, especially in ruptured spores.

Spores were exposed to sodium hydroxide by the method previously described. After neutralizing, the spore suspensions were centrifuged at 4,300 x g for 10 min. and washed once in distilled water. After resuspending the spores in distilled water the suspension was allowed to come to room temperature. A drop of the spore suspension was placed on a chemically cleaned glass slide and a drop of 2% aqueous PTA (pH 7.2) was mixed with it for 30 sec. A loopful of the stained suspension was

placed on a carbon-coated grid; the spores allowed to settle for one min., the excess fluid withdrawn with a filter paper wick, but without completely drying the grid. The grid was then air-dried for at least one min. and examined in the electron microscope. Unstained spores in distilled water were examined as a control to determine if the treatment had resulted in any visible changes.

Preparation of Sectioned Material

Fixing The neutralized spore suspension was centrifuged at 4,300 x g for 8 min., the supernatant fluid discarded, and the spores washed once with distilled water. The following solutions were prepared:

- (1) Veronal acetate buffer (Palade, 1952)

Barbitone sodium	2.94g
hydrated sodium acetate	1.94g
NaCl	3.40g
- brought to 100 ml.	
with distilled water	

- (2) Kellenberger's buffer

Veronal acetate buffer	5.0 ml.
distilled water	13.0 ml.
0.1N HCl	7.0 ml.
M - CaCl ₂	0.25 ml.

- (3) Kellenberger's OsO₄ fixative

OsO ₄	0.1 g
Kellenberger's buffer	10.0 ml.

- (4) Washing Solution

uranyl acetate	0.5 g
Kellenberger's buffer	100 ml.

- (5) "Tryptone" medium

Bacto tryptone	1.0 g
NaCl	0.5 g
distilled water	100 ml.
- autoclaved at 121°C for 15 min.	

(6) Buffered agar

2% Difco's Special "Noble" Agar
prepared in Kellenberger's buffer
rather than in water.

The spores were fixed by overnight suspension at room temperature in a 100:1 mixture of Kellenberger's OsO_4 fixative and the Bacto tryptone medium.

After fixation the spore - fixative mixture was diluted with Kellenberger's buffer (fixative:buffer = 1:8) and centrifuged to recover the spores. The spore pellet was mixed with a drop of buffered agar. By decanting this mixture onto a chemically cleaned cold glass slide small pellets were formed. These pellets were minced with a cleaned razor blade and the resulting cubes placed in the uranyl acetate washing solution for 2 hr. at room temperature.

Epon Embedding The washed fragments of the pellet were dehydrated by immersion, for 15 min. in each of the following solutions (% v/v) of ethanol: 35%, 50%, 70%, 95%, and 100%, and were finally placed in a second 100% ethanol bath while the resins were being prepared.

An epoxy resin was prepared as follows (Luft, 1961):

Epon 812(1)	62 ml.)	) mixture A
DDSA(2)	100 ml.)	
Epon 812	100 ml.)	) mixture B
NMA(3)	89 ml.)	

- (1) - a glycerol based, aliphatic epoxy resin developed by Shell Chem. Co.
- (2) - dodecenyl succinic anhydride
- (3) - nadic methyl anhydride

Mixtures A and B, after very thorough mixing, were subjected to low vacuum for 30 min. to remove air, and stored at 4°C.

A resin mixture which, on polymerization gave a block of the desired hardness, was prepared by mixing 3 ml. of mixture A and 7 ml. of mixture B and adding 0.15 ml. of the accelerator, AMP - 30₍₄₎.

Fragments were removed from the 100% ethanol and immersed in propylene oxide and graded resin mixtures in the following sequence: propylene oxide for 15 min., propylene oxide for 15 min., propylene oxide:resin mixture (1:1) for 1 hr. with agitation, propylene oxide:resin mixture (1:3), left overnight at room temperature. Following this the specimens were transferred to gelatin capsules containing fresh resin mixture, with accelerator, and these polymerized by heating at 37°C for 12 hr. followed by 60°C for 72-96 hr.

The resultant blocks were trimmed, fitted to a hand-operated Porter Blum microtome, and "gray" sections (600-900 Å thick) were cut using a glass knife. The sections were floated on a 5% acetone solution, transferred to carbon coated grids, and examined and photographed in a Philips EM100 electron microscope.

Vestopal-W Embedding The epoxy resin, Vestopal-W₍₁₎, forms a somewhat finer-grained matrix than do the methacrylate resins or Epon 812 and, according to Fitz-James (1960) makes possible somewhat better resolution of cell layers. Accordingly, Vestopal-W supported sections of spores were compared with those in Epon 812.

(4) - 2,4,6 - tri dimethylaminomethyl phenol

(1) - epoxy resin manufactured by M. JAEGER, VESENEZ, SWITZERLAND

Spores were fixed as previously described, washed in uranyl acetate, and dehydrated according to the following schedule, which is modified from that of Kellenberger et al. (1958). Dehydration was by immersion in each of the following solutions (% v/v) of acetone: 30%, 50%, and 75%, for 30 min. each; 90% and 100% for 60 min. in each and a second bath in 100% for 60 min.

The fragments were removed from the 100% acetone and put through propylene oxide and graded resin mixtures in the following sequence, also adapted from Kellenberger et al.: propylene oxide for 10 min., propylene oxide:resin (3:1) for 30 min., propylene oxide:resin (1:1) for 30 min., propylene oxide:resin (1:3) for 30 min., resin (with accelerators) overnight, with agitation at room temperature. Following this the specimens were placed in gelatin capsules, fresh resin with accelerators added, and polymerization effected by heating at 59-60°C overnight.

Gray sections were cut (600-900 Å in thickness), using a Du Pont diamond knife, on an automatic Porter Blum (MT-2) microtome. Sections were floated on 5% acetone and transferred to carbon coated 250-mesh copper grids. They were examined in the electron microscope.

RESULTS

The results of the plate colony counts which were made on samples used for electron microscopy have been averaged and expressed as semi-logarithmic disinfection curves in Figs. 4, 5 and 6. The percentage kill of spores, as shown by these curves, has been used to assist in interpreting the effects of the disinfectant treatments noted visually and by photography with the electron microscope.

Whole Mounts

Whole mounts were made of untreated controls, i.e. spores held in distilled water at temperatures and times similar to those used in the disinfection processes, and also of spores exposed to two different disinfection treatments. All specimens were stained negatively with phosphotungstic acid, by the method previously described.

Controls

Fig. 7 is typical of whole mounts of the untreated controls. The spores are completely opaque to the electron beam.

Figs. 8 and 9 show examples of collapsed and ruptured spores. These were rare, in both control and treated specimens, indicating that the preparation of specimens did not cause any appreciable damage to the spores.

Treated Spores

(a) 1.5% NaOH at 70°F (21.1°C) for 13 hr.

Plate counts of the spore populations from which these samples were taken indicated that 99.9% of the spores were non-viable.

Fig. 10 is typical of what was observed with these treated spores. The spores were opaque, and in most cases no evidence of damage was to be seen.

A few spores showed evidence of what appeared to be a lifting away of the outer spore wall layer. This is illustrated in Fig. 11.

(b) 5% NaOH at 70°F (21.1°C) for 13 hr.

Samples from this spore population, when plated, were 99.99% non-viable.

Fig. 12 shows a spore typical of those observed after this disinfection treatment. Such spores were opaque and showed no evidence of damage by the treatment.

A few spores resemble that shown in Fig. 13. They appear to be ruptured at one pole, although the fact that they did not show reduced opacity after rupture (cf. Figs. 8 and 9) suggests that the figure may be a whole spore with one end encased in electron dense debris.

Sectioned Material

Initial attempts to use Epon were notably unsuccessful. It became clear, after allowing for the author's inexperience, that this resin did

not readily penetrate beyond the cortex in the spores of the species used. The lack of specimen support resulted in smearing of the spore core during sectioning, and obliteration of all structural details of the spores. Longer pre-embedding and polymerization times improved the quality of embedding but the results were never as predictable as were those obtained with Vestopal-W.

Sections were cut from both Epon and Vestopal-W blocks. The Epon mounted specimens were slightly more difficult to cut, and gave a darker background than the Vestopal-W, which resulted in a reduction in contrast in these sections. Sections were made of untreated controls and of spores exposed to three different disinfection treatments.

Controls

Figs. 14, 15 and 16 indicate the structure of untreated spores of B. subtilis (cf. Fig. 3). Figs. 14 and 16 are from sections cut from Epon, Fig. 15 is from a section from a Vestopal-W block.

All of the structures normally observed in sections of B. subtilis spores can be seen in Fig. 14. In Fig. 15 the laminated nature of the middle layer of the spore wall and of the cortex can be seen. In Fig. 16 the ridged nature of the outer coat of the spore is evident. This illustration is probably a cross-section through the longitudinal ridges observed by Bradley and Williams (1957), on spores of B. subtilis.

Treated Spores

(a) 1.5% NaOH at 50°F (10°C) for 36 and 72 hr.

This treatment was intended as a slow disinfection process. Samples were taken at 36 and 72 hr. and embedded in Epon. Plate counts at 36 hr. showed that 96% of the spores were non-viable, and at 72 hr. over 99% of the spores failed to germinate.

Fig. 17 shows a spore typical of those examined after 36 hr. exposure to the disinfectant. There is some evidence of breakdown of the cortex. The area marked 'x' is probably an artifact produced by "enucleation" by the knife during sectioning.

Figs. 18 and 19 show typical sections of specimens exposed to the disinfectant for 72 hr. These show a featureless spore core and either shrinking away or dissolution of the cortex. While the resolution of these photomicrographs is not the best, it would appear that the spore coats were intact.

(b) 1.5% NaOH at 70°F (21.1°C) for 13 hr.

This treatment provided a higher rate of disinfection than that described in (a) above. After 12 hr. exposure the spore population, on plating on SMA, was 99.9% non-viable.

Figs. 20, 21 and 22 are typical of the spore sections examined. There is some evidence of collapsing spore walls and elongation, or extrusion, of the spore cores. In Fig. 20 the spore core appears to merge with the spore wall, suggesting rupture of the spore coats and subsequent extrusion of the spore contents.

In Figs. 21 and 22 the poorly defined cortex and elongated, poorly defined spore core indicate that the cortex and spore core coat have been disorganized.

(c) 5% NaOH at 70°F (21.1°C) for 13 hr.

This treatment provided a relatively drastic disinfection process. Plate counts showed that 99.99% of the spores were non-viable after 13 hr.

In view of this high percentage of non-viable spores the appearance of these samples was, at first sight, surprising. Approximately half of the sections observed appeared to be intact, whereas the remainder showed the fairly gross damage expected.

Fig. 23 shows a spore with structure largely intact. There is, however, a suggestion of internal damage; the arrow at 'x' indicates a large interspace between the inner coat of the spore wall and the outer cortical membrane. This could be formed by expansion of the spore wall, or more likely, by shrinkage of the cortex. Within this interspace the layers of the spore wall have, in some areas, become disarranged, perhaps pulled in by a shrinking cortex.

Figs. 24 and 25 show, respectively, early and late stages in the rupture and loss of contents shown by about 50% of the spores subjected to this treatment. The collapsing spore core in Fig. 24 is noteworthy.

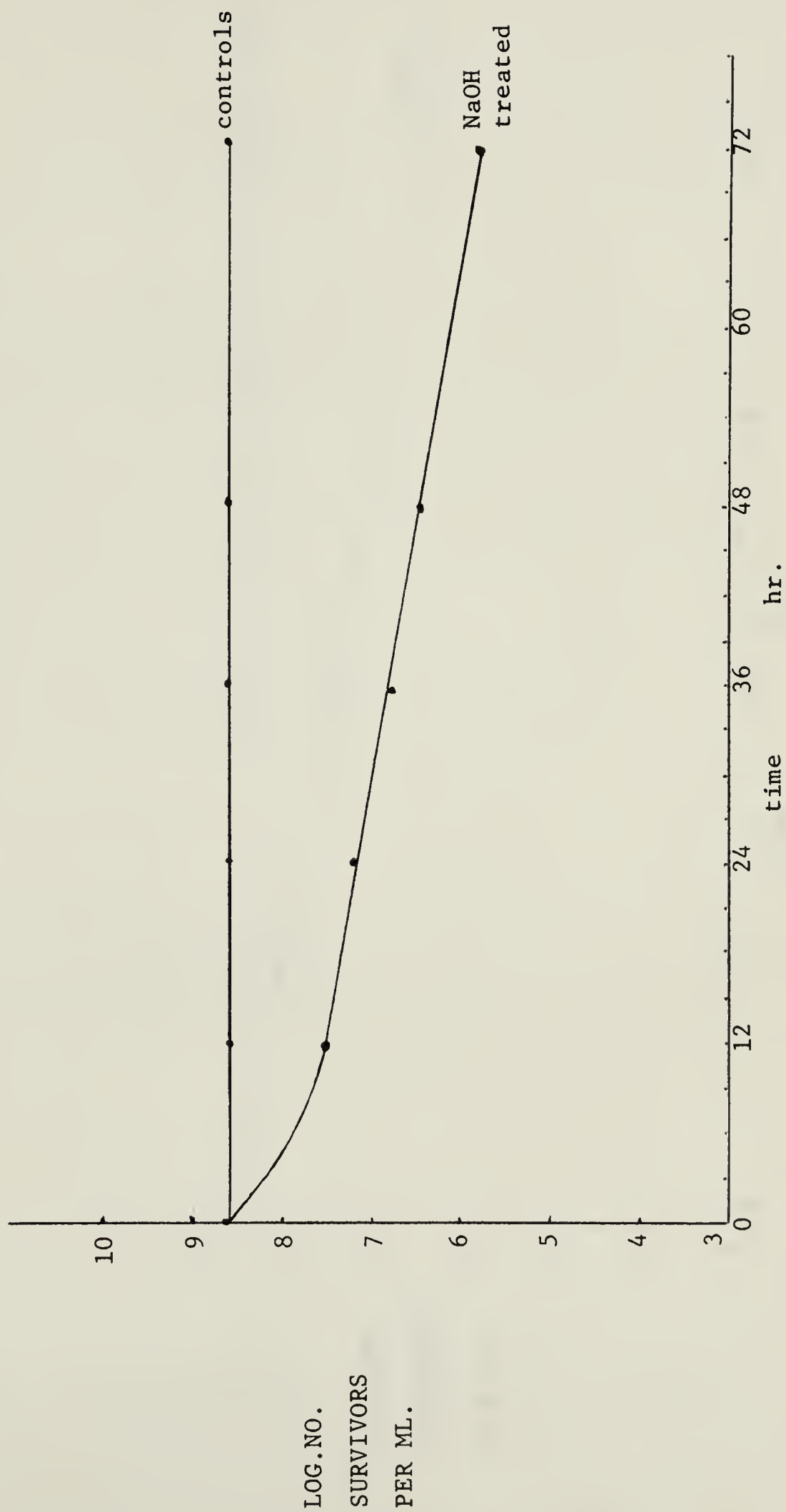


FIG. 4 The Effect of 1.5% NaOH on *B. subtilis* Spores at Temp. 50°F (10°C)

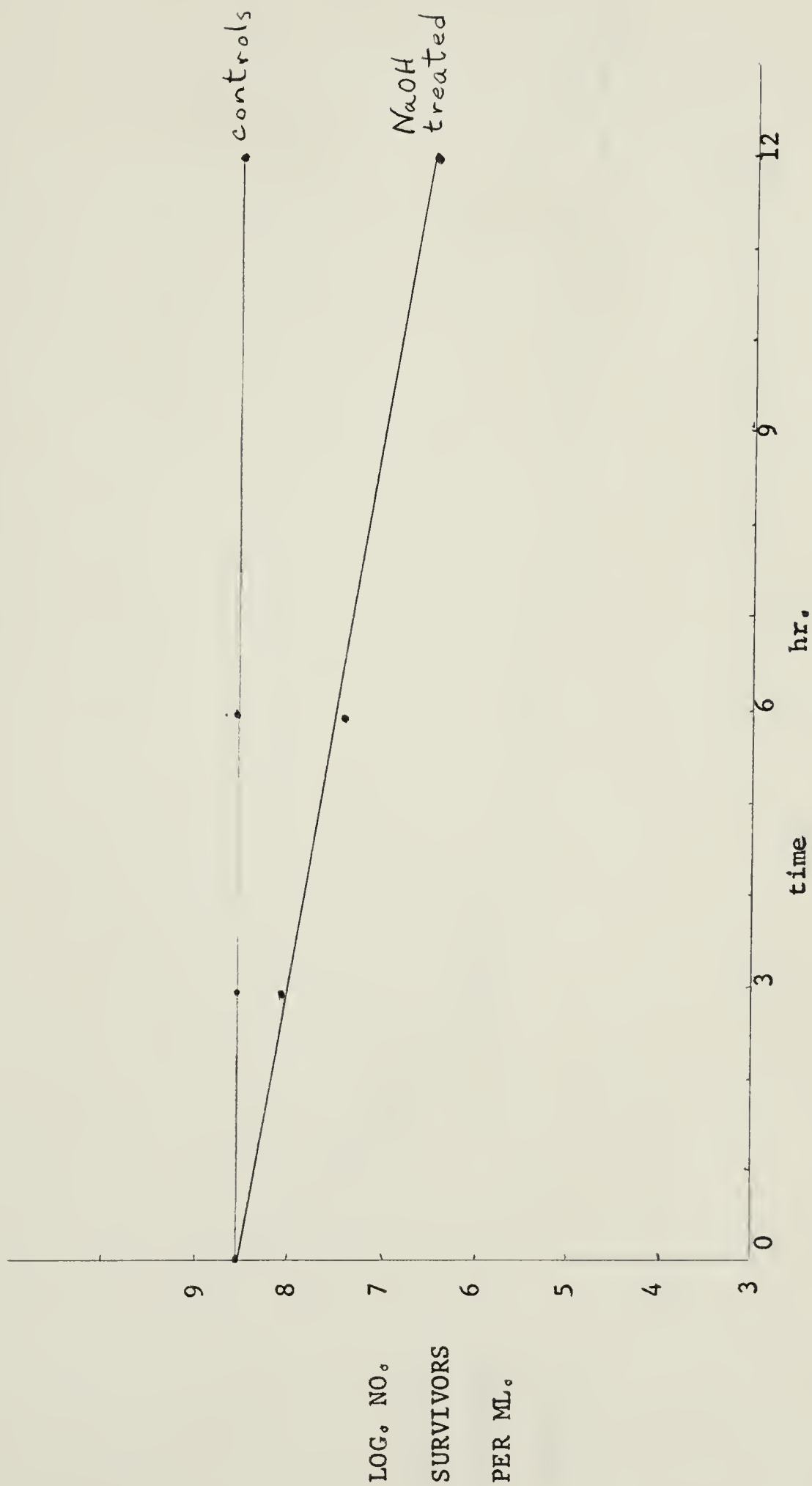


FIG. 5 The Effect of 1.5% NaOH on B. subtilis Spores at Temp. 70°F (21.1°C)

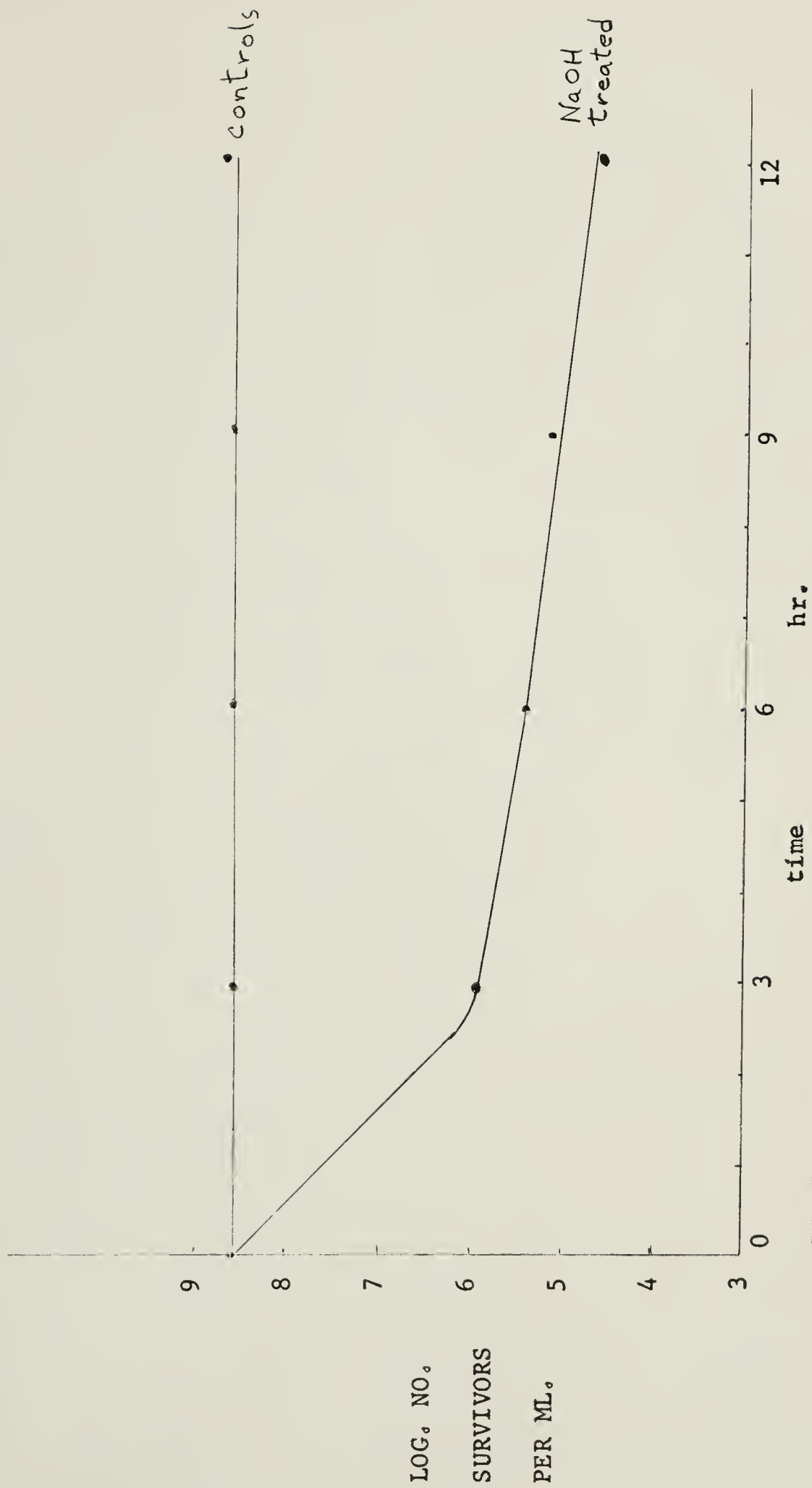


FIG. 6

The Effect of 5% NaOH on *B. subtilis* Spores at
Temp. 70°F (21.1°C)

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Fig. 7. B. subtilis spores suspended in distilled water.
Whole mount, stained with phosphotungstic acid.
(x 36,500).



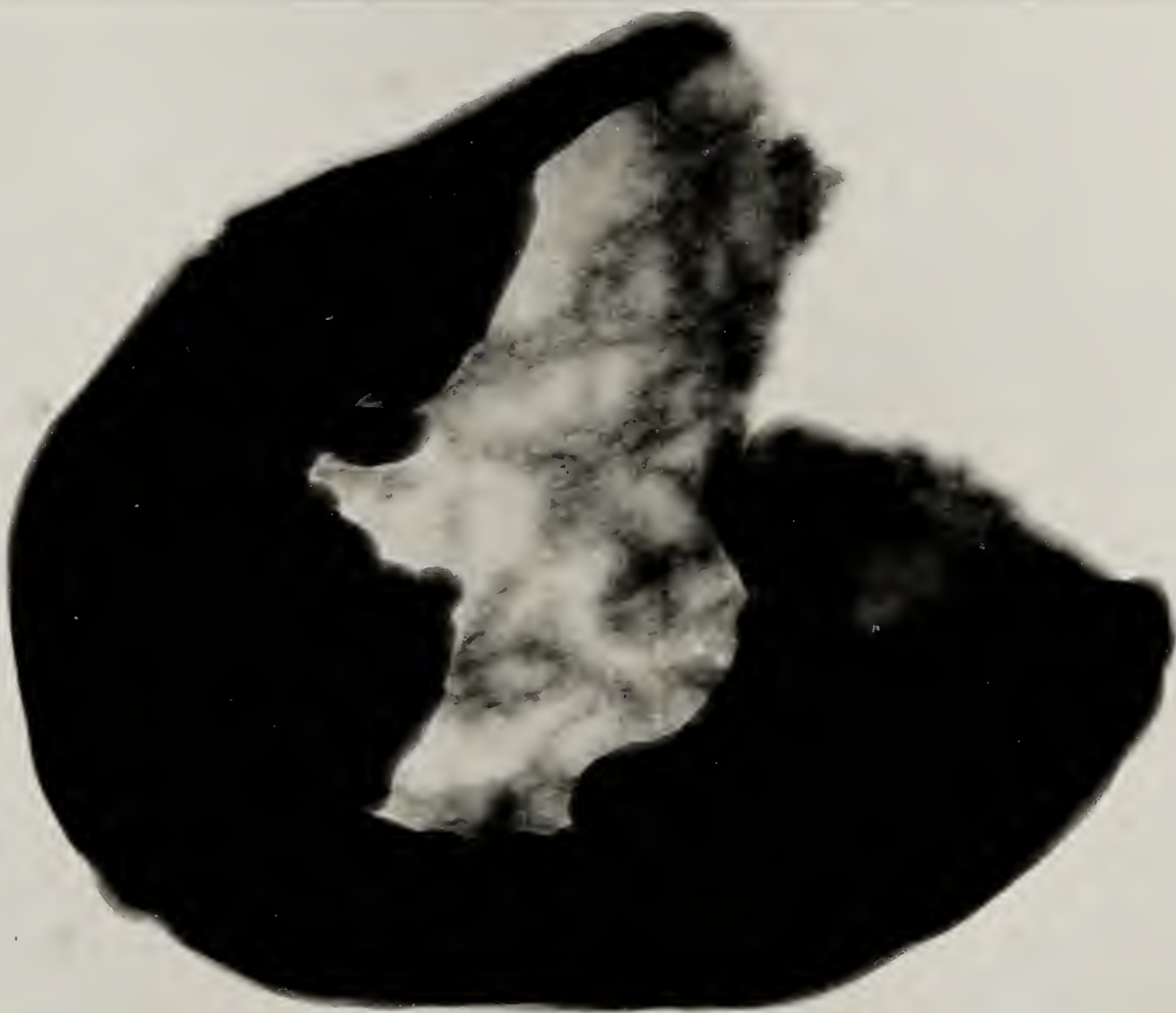
(to face page 55)

Fig. 8. B. subtilis spores suspended in distilled water.
Whole mount, stained with phosphotungstic acid.
(x 57,500).

Fig. 9. B. subtilis spores suspended in distilled water.
Whole mount, stained with phosphotungstic acid.
(x 55,000).



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Fig. 10. B. subtilis spores treated with 1.5% NaOH at 70°F for 13 hr.
Whole mount, stained with phosphotungstic acid. (x 67,000).

Fig. 11. B. subtilis spores treated with 1.5% NaOH at 70°F for 13 hr.
Whole mount, stained with phosphotungstic acid. (x 41,000).
Arrows (a) mark areas where outer layers of spore wall
appear to be lifted away.



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Fig. 12. B. subtilis spores treated with 5% NaOH at 70°F for 13 hr.
Whole mount, stained with phosphotungstic acid. (x 78,000).

Fig. 13. B. subtilis spores treated with 5% NaOH at 70°F for 13 hr.
Whole mount, stained with phosphotungstic acid. (x 63,500).
Arrows (a) mark area of possible rupturing of the spore
wall. (Note: the possibility that the marked areas are
masses of electron-dense debris capping an undamaged spore
has not been wholly excluded.)



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Fig. 14. B. subtilis spores suspended in distilled water.
Section cut from Epon block (x 64,000).

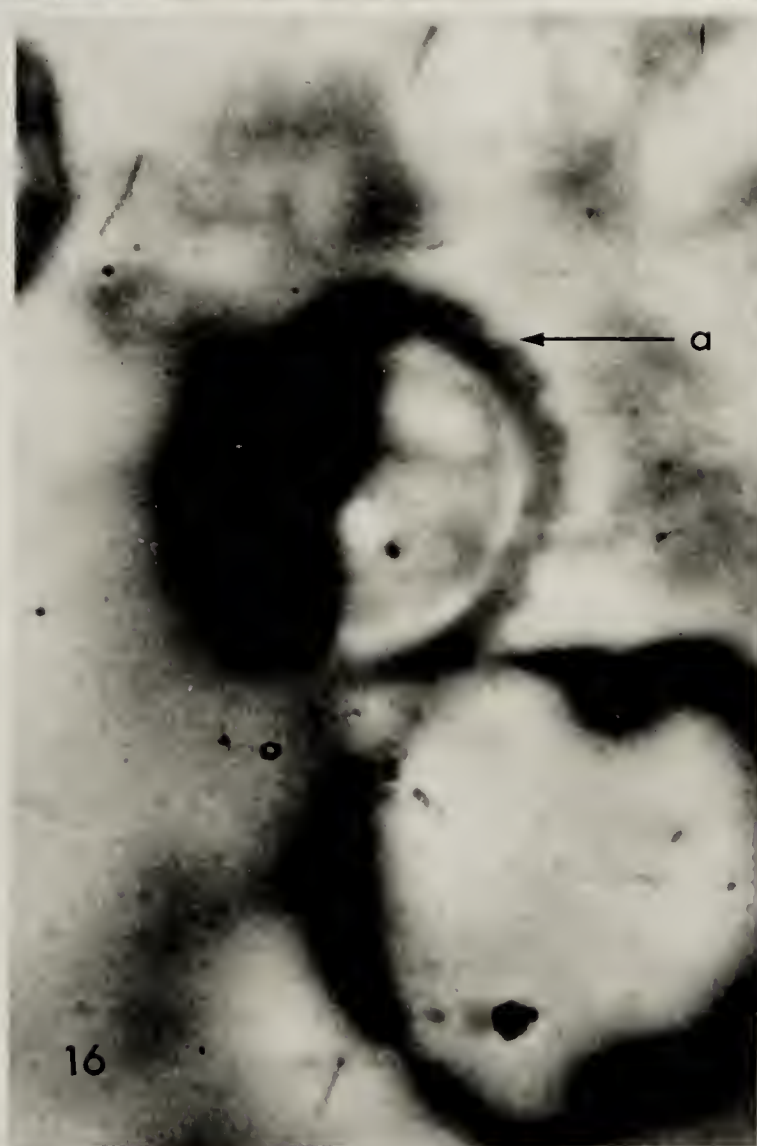
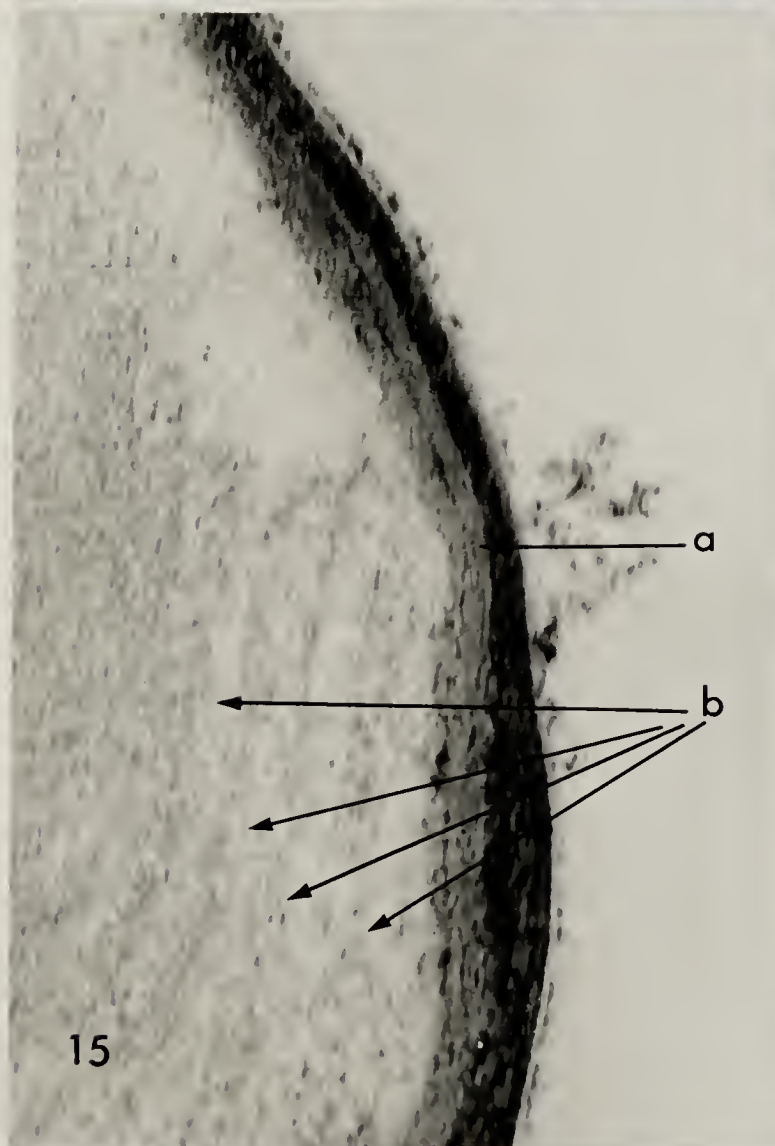
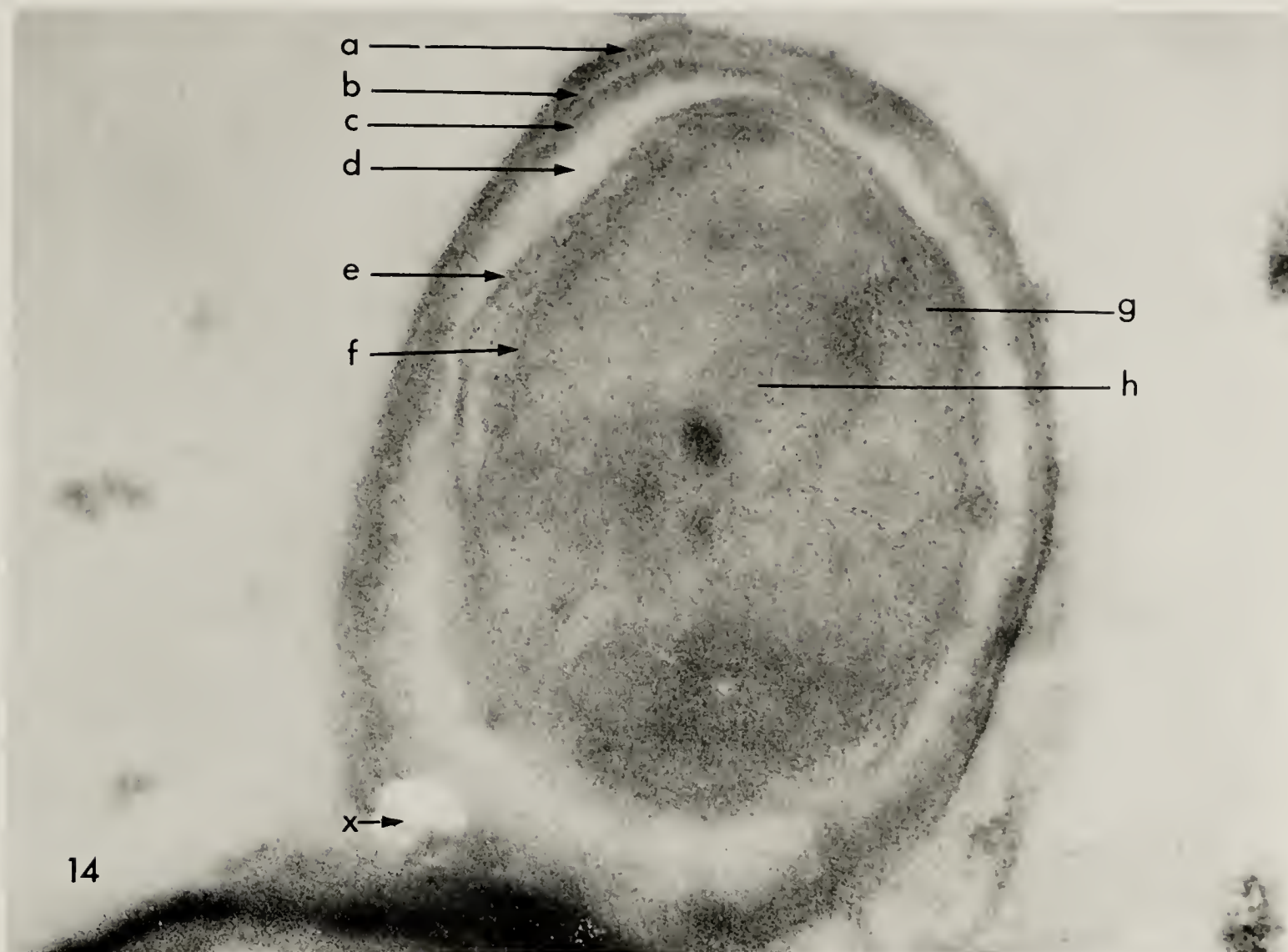
(a) outer coat of spore wall, (b) laminated layer of spore wall (cf. Fig. 15), (c) inner coat of spore wall, (d) cortex, (e) spore core coat, (f) spore core cytoplasmic membrane, here pulled away from spore core coat, (g) spore nuclear material (note fibrillar nature), (h) spore cytoplasm, (x) artifact produced by the electron microscope.

Fig. 15. B. subtilis spores suspended in distilled water.
Section cut from Vestopal-W block. (x 118,000).

(a) laminated layer of spore coat, (b) laminations of cortex.

Fig. 16. B. subtilis spores suspended in distilled water.
Sections cut from Epon block. (x 57,500).

Note the characteristic external ridges displayed by this species (a).



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Fig. 17. B. subtilis spores treated with 1.5% NaOH
at 50°F for 36 hr.

Section cut from Epon block. (x 52,000).

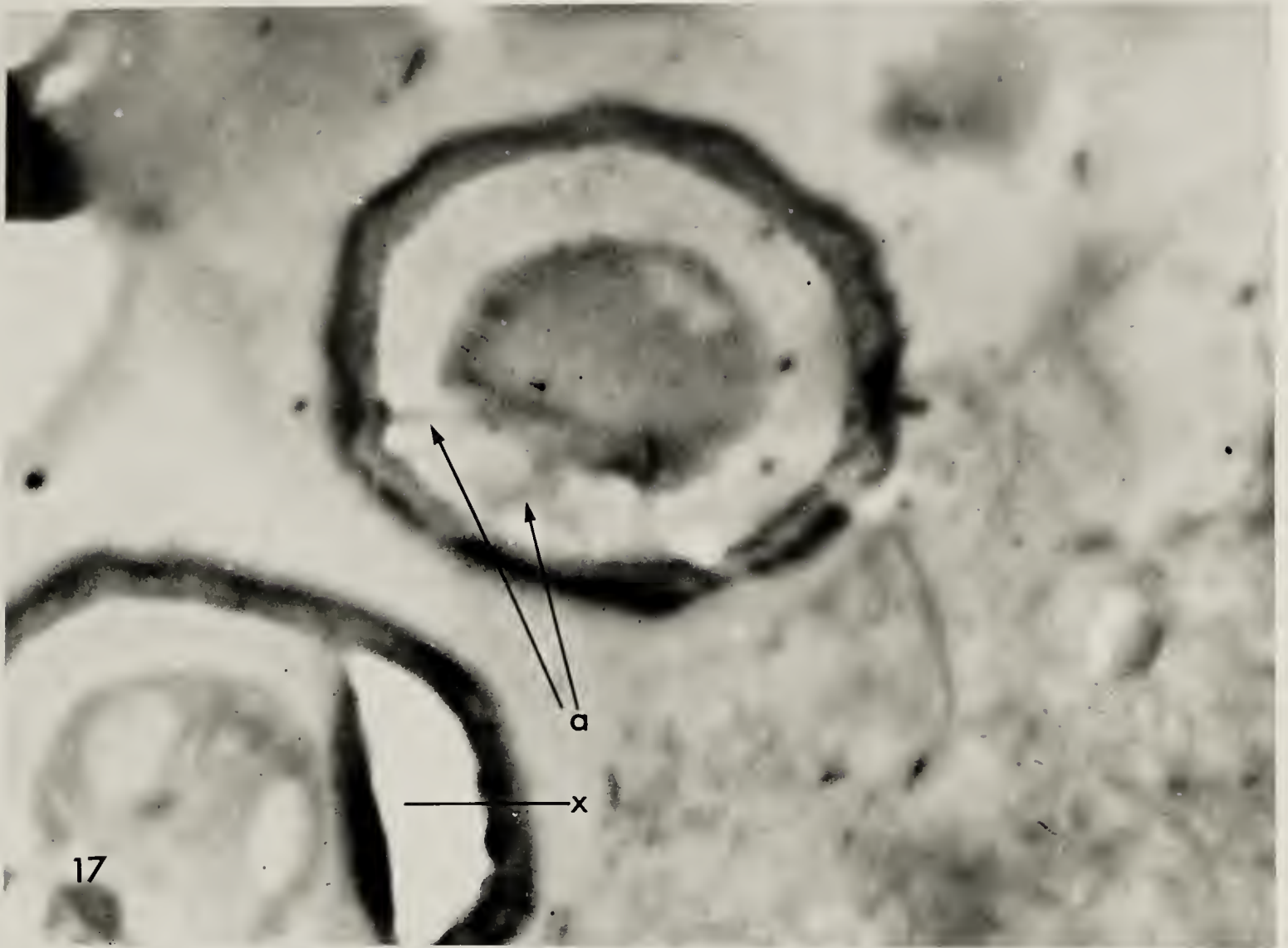
Note the disarrangement of the cortex at (a)
and the "enucleation" at (x).

Fig. 18. B. subtilis spores treated with 1.5% NaOH
at 50°F for 72 hr.

Sections cut from Epon block. (x 50,500).

Fig. 19. B. subtilis spores treated with 1.5% NaOH
at 50°F for 72 hr.

Sections cut from Epon block. (x 50,500).



(to face page 65)

- Fig. 20. B. subtilis spores treated with 1.5% NaOH
at 70°F for 13 hr.
Section cut from Vestopal-W block. (x 63,000).
Note the merging of the spore core with the
area of the spore wall at (a) and the
collapsing area of spore wall at (b).
- Fig. 21. B. subtilis spores treated with 1.5% NaOH
at 70°F for 13 hr.
Sections cut from Vestopal-W block. (x 63,000).
- Fig. 22. B. subtilis spores treated with 1.5% NaOH
at 70°F for 13 hr.
Sections cut from Vestopal-W block. (x 54,000).

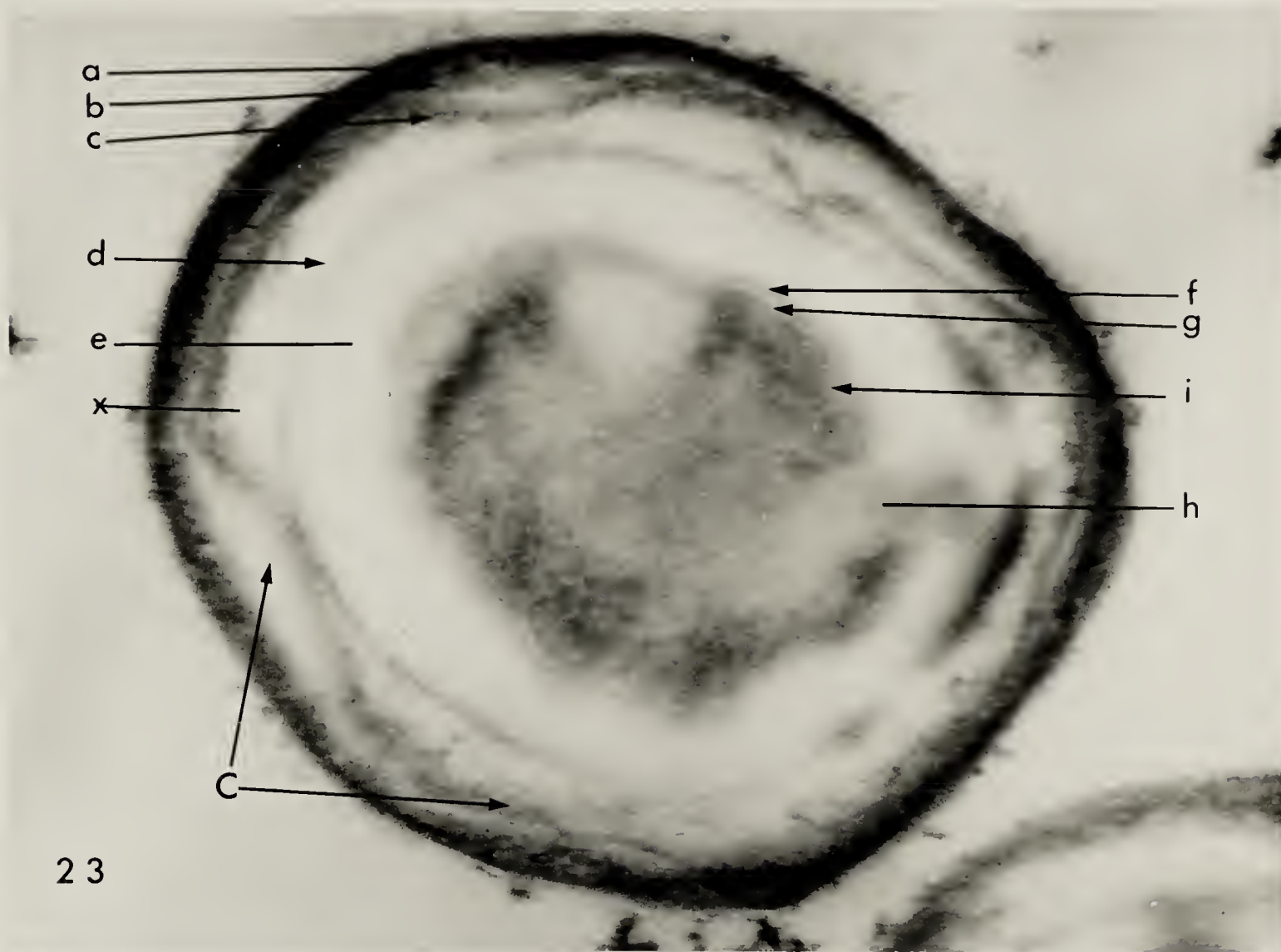


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Fig. 23. B. subtilis spores treated with 5% NaOH
at 70°F for 13 hr.
Section cut from Vestopal-W block. (x 65,500).
The following are indicated: (C) coats of
the spore wall in a large interspace,
(a) outer coat of the spore wall, (b) laminated
layer of the spore wall, (c) inner coat of the
spore wall, (d) cortical membrane, (e) cortex,
(f) spore core wall, (g) spore core cytoplasmic
membrane, (h) spore cytoplasm, (i) spore nuclear
material, (x) interspace between spore wall and
cortical membrane.

Fig. 24. B. subtilis spores treated with 5% NaOH
at 70°F for 13 hr.
Section cut from Vestopal-W block. (x 39,500).
Note collapsed spore core (S).

Fig. 25. B. subtilis spores treated with 5% NaOH
at 70°F for 13 hr.
Section cut from Vestopal-W block. (x 37,500).
More advanced rupturing and loss of contents
than in Fig. 24.



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DISCUSSION

The data, and the techniques used, are conveniently discussed under two subheadings:

- (1) possible sources of error and/or omission in the collection of data
- (2) the meaning of the data per se.

Possible Sources of Error

The disinfection curves (Figs. 4, 5 and 6) clearly show that a large percentage of the spore population was non-viable, in every case well before samples were taken for electron microscopy. If samples of treated spores had been taken at earlier stages in the disinfection process this would have reduced the possibility of post mortem damage obscuring the changes which indicated the cause of death.

Plate counts by themselves do not indicate the differences between killed spores and those rendered temporarily incapable of germinating. In those experiments where plate counts showed a high percentage of non-viable spores, but electron micrographs showed only moderate damage, it is possible that many of the spores had not been permanently affected. Thorough washing of such treated spores, and cultivation in "recovery media" might show if such spores, which had failed to germinate after coagulation of spore coat protein, had failed because they were merely impermeable to germinating agents.

At present, the interpretation of electron micrographs is a rather personal function. Several current workers (see Harris, 1962) have seriously discussed the dangers of misinterpretation in a system where the researcher is required, first, to study and record only small samples of a relatively large amount of material, and second, to interpret this as applying to a whole population. Further, there is a tendency, in interpreting photographs, to accept these as being representative of the material in the living, or pre-fixed form. It is difficult to accept, as artifact, a picture which supports a theory, and it is easy to reject, as artifact, a piece of apparently contradictory evidence. In this field, perhaps more than any other, the researcher should, therefore, be his own severest critic.

Suggestions from the Data per se

Whitehouse (1961) and earlier workers used starch milk agar (SMA) as a recovery medium for spores of B. subtilis which had been exposed to sodium hydroxide solutions. In the present study the temperatures, exposure times, and concentrations of alkali were those used by Whitehouse, as was the strain of B. subtilis, in its spore state. The data of Whitehouse and the data presented in this study as graphs agree very closely.

Observations of whole mounts, although somewhat unrewarding, showed that ruptured spores were observed as often with untreated controls as with treated spores. This observation suggests that the

rupturing may have been a result of surface tension forces acting on the specimens during drying, rather than of the disinfection treatment.

Since the opacity of spores is reduced appreciably when loss of contents occurs (cf. Figs. 8 and 9) this might be a useful criterion in deciding whether rupture has occurred in whole mounts of spores. For example, initial inspection of Fig. 13 suggests that the spore is ruptured. The complete opacity of the spore, however, admits another interpretation; the outer coat of the spore wall may be lifted away and, capped with electron-dense debris, may give the appearance of extruded material.

Sections reveal evidence of gross protein denaturation, particularly of the spore cortex. Although the sections were from samples taken late in the disinfection process, it is possible to infer from Fig. 17, in contrast to Fig. 18, and from Figs. 20, 21 and 22, in comparison with Fig. 23, that the cortex is broken down more rapidly than are the spore coats, and that disarrangement of the cortex precedes damage to the spore core.

Comparison of Figs. 19 and 23, which were from populations where the plate counts showed less than 1% viable spores, suggests that the observable gross damage done to spores by sodium hydroxide solutions may be more a function of contact time than of disinfectant concentration, and that there may be critical concentrations of sodium hydroxide above which observable damage is fairly uniform, although the rate of

kill had increased.

This could also mean that the physical damage required to render a spore incapable of germination may not be gross enough to be observed but may, for example, make the spore wall impermeable to germinating agents. Any subsequent damage might then be regarded as "post mortem" change. Such a situation would assist in explaining the large numbers of spores which appeared to be relatively intact (cf. Fig. 23) yet which are known, from plate colony counts, to have been 99.99% non-viable, after exposure to 5% sodium hydroxide. The phenomenon of these apparently intact spores may be partially explained, also, by the possibility that the 5% sodium hydroxide caused a very rapid denaturation of the protein of the spore wall. If coagulation were rapid enough the altered spore wall might be temporarily impermeable to further penetration of the disinfectant. The result could be preservation of a relatively intact spore which, at the same time, would be impermeable to germinating agents and would be non-viable according to plate counts. Longer exposure would have resulted in further gross destruction but, perhaps in the time interval used (i.e. 13 hr.), this form of limited denaturation could exist.

The initial work of Powell (1957 b) and others indicates that an initial step in the germination of B. subtilis spores is loss of a DAP-hexosamine peptide from the spore wall, along with the appearance of a lysozyme - like enzyme capable of releasing this peptide from spore wall protein, and release of calcium dipicolinate. The release of

Ca-DPA may initiate further germination changes by releasing enzymes which had been stabilized and inactivated by chelation with the calcium of the compound. Whatever its mode of action, however, Ca-DPA must be released before germination proceeds, and it seems that this release may be made possible by an alteration of the spore wall protein by loss of a peptide. Anything which would prevent an alteration of spore wall permeability and, therefore, prevent the release of Ca-DPA, would prevent germination. These suggestions appear to the author to relate to the unexpected phenomenon discussed above, of strong alkali rendering spore populations almost completely non-viable, without appropriate evidence of physical destruction.

It would be necessary to undertake a more intensive study of the disinfection processes described herein, particularly during that part of the process where the percentage kill of spores is low, before this "morphological" method of studying disinfection of spores could be further assessed.

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